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**THE GLUCOREGULATORY ACTION OF
GLUCAGON-LIKE PEPTIDE-1 (GLP-1).**

by

Malgorzata Adamczyk

**A thesis submitted to the School of Graduate Studies and Research of the University
of Ottawa in partial fulfilment of the requirements for the degree of Master of Science.**

Department of Physiology

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Ottawa, Canada

May, 1995.

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) has been shown to improve tolerance to glucose. It has been suggested that this could be mediated by an incretin effect – the enhancement of insulin secretion in response to glucose, as well as by alterations in the sensitivity of the body to insulin. In order to evaluate the effect of GLP-1 on the improvement of glucose tolerance, the systemic as well as tissue-specific (the liver, intestine and muscle) effects of this hormone have been determined.

The study has been conducted on animal model (the pig). Following an overnight fast and baseline measurements, glucose was infused (set point = 150 mg/dl), alone or supplemented with GLP-1 (4 ng/kg/min) and GLP-1 (8 ng/kg/min) in 90 min steps. The levels of metabolites (glucose, lactate) and hormones (insulin, glucagon, GLP-1) were then determined in arterial blood as well as in portal, hepatic and femoral venous blood. Tissue balances were then calculated. Levels of metabolites and hormones, glucose infusion rates and tissue balances were compared using statistical analysis (general linear models procedure, SAS Institute).

The results of the study revealed that during glucose infusion the blood levels of physiologically secreted GLP-1 did not change (89.3 ± 21.5 versus 62.4 ± 16.3). Supplementary administration of GLP-1 resulted in increases in concentration of this hormone (312 ± 21 pg/ml during the 4 ng/kg/min infusion, and 565 ± 58 pg/ml during the 8 ng/kg/min infusion). The amount of glucose required to maintain the level of hyperglycemia was linked to the administration of GLP-1, culminating in an almost two-fold increase during administration of 8 ng/kg/min of GLP-1, as compared to control. Simultaneously, insulin concentrations in-

creased from 9.66 ± 1.07 $\mu\text{U/ml}$ (average basal) to 23.67 ± 6.2 $\mu\text{U/ml}$ (control: 14.03 ± 3.6 $\mu\text{U/ml}$) during administration of 4 ng/kg/min of GLP-1 and to 27.42 ± 6.8 $\mu\text{U/ml}$ (control 16.07 ± 5.99 $\mu\text{U/ml}$) during infusion at the rate of 8 ng/kg/min.. The insulinotropic action of GLP-1 was confirmed by direct measurement of C-peptide concentration.

Metabolite balances across representative tissues: the splanchnic bed (intestine and liver) and periphery (muscle of hindlimb) were also examined. The liver was transformed from glucose-producing (2.3 ± 0.4 mg/kg/min) to a glucose-extracting organ during the infusion of glucose alone (0.6 ± 0.5 mg/kg/min). The addition of GLP-1 at 4 ng/kg/min (1.1 ± 0.3 mg/kg/min) or at 8 ng/kg/min (0.9 ± 2.1 mg/kg/min) did not significantly alter this glucose extraction. Intestinal glucose uptake demonstrated a similar lack of sensitivity to the addition of GLP-1. The glucose uptake in the hindlimb increased from 0.07 ± 0.025 mg/kg/min to 0.15 ± 0.03 mg/kg/min and to 0.45 ± 0.10 mg/kg/min and 0.50 ± 0.08 mg/kg/min during the administration of 4 ng/kg/min and 8 ng/kg/min of GLP-1 respectively.

The study demonstrates that:

- (1) The incretin effect (enhancement of insulin secretion) is reproduced in this animal model.
- (2) Increases in glucose uptake during the infusion of GLP-1 can be explained by the insulin effect alone .
- (3) There is no enhancement of insulin action by GLP-1 from the systemic perspective.
- (4) The enhancement of glucose metabolism induced by GLP-1 and mediated by changes in insulin occurs primarily at the level of muscle and not the splanchnic bed.

TABLE OF CONTENTS

I. Abstract	i
II. Table of contents	iii
III. List of figures	vi
IV. Acknowledgements	vii
1. Introduction	1
2. Materials and methods	28
2.1. Animals	28
2.1.1. Animals and housing	28
2.1.2. Surgery	28
2.2. Experimental procedures	30
2.2.1. Objectives	30
2.2.2. Preparation of the infusates	30
2.2.2.1. Indocyanine green (ICG)	30
2.2.2.2. Glucagon-like peptide-1 (GLP-1)	30
2.2.2.3. Glucose	31
2.2.3. Experimental protocol	31
2.2.3.1. Sampling	32
2.2.3.2. Treatment of blood samples	32

2.2.4. Determination of blood (plasma) flow	33
2.2.4.1. Femoral vein blood flow	33
2.2.4.2. Indocyanine green assay	33
2.2.4.3. Liver plasma flow	34
2.2.5. Determination of the concentrations of metabolite in plasma	34
2.2.5.1. Glucose concentration	34
2.2.5.2. Lactate concentration	35
2.2.6. Determination of the concentrations of hormone in plasma	35
2.2.6.1. Basic principles of the radio-immunoassay technique	35
2.2.6.2. Insulin radioimmunoassay	37
2.2.6.3. C-peptide radioimmunoassay	38
2.2.6.4. Glucagon-like peptide-1 radioimmunoassay	39
2.2.6.5. Glucagon radioimmunoassay	39
2.3. Calculations	40
2.3.1. Assessment of tissue blood (plasma) flow	40
2.3.2. Tissue fluxes of metabolites and hormones	41
2.3.2.1. Liver balances	42
2.3.2.2. Splanchnic balances	42
2.3.2.3. Intestinal balances	43

2.3.2.4. Muscle balances	43
2.3.3. Assessment of insulin secretion	44
2.3.4. Data analysis and statistics	44
2.3.4.1 Comparison of arterial metabolite and hormone concentrations	44
2.3.4.2. Comparison of tissue balances	45
3. Results	47
3.1. Metabolite concentrations	47
3.2. Hormone concentrations	50
3.3. Glucose requirements	56
3.4. Tissue balances of metabolites and hormones	58
3.4.1. Glucose balances	58
3.4.2. Lactate balances	63
3.4.3. C-peptide balances	66
3.4.4. Insulin balances	68
4. Discussion	70
5. Conclusions	78
6. References	79

LIST OF FIGURES

- Figure 1.** Average plasma glucose concentrations in controls and GLP-1 treated animals.
- Figure 2.** Average plasma lactate concentrations in controls and GLP-1 treated animals.
- Figure 3.** Average plasma GLP-1 concentrations in controls and GLP-1 treated animals.
- Figure 4.** Average plasma C-peptide concentrations in controls and GLP-1 treated animals.
- Figure 5.** Average plasma insulin concentrations in controls and GLP-1 treated animals.
- Figure 6.** Average plasma glucagon concentrations in controls and GLP-1 treated animals.
- Figure 7.** Average rates of glucose infusion for controls and GLP-1 treated animals.
- Figure 8.** Glucose uptake by the splanchnic bed and liver in controls and GLP-1 treated animals.
- Figure 9.** Glucose uptake by the intestinum and muscle in controls and GLP-1 treated animals.
- Figure 10.** Lactate extraction by the splanchnic and liver tissues in the controls and GLP-1 treated animals.
- Figure 11.** Lactate uptake by the intestinal and muscle tissues in the controls and GLP-1 treated animals.
- Figure 12.** C-peptide uptake by the splanchnic bed in the controls and GLP-1 treated animals.
- Figure 13.** Insulin uptake by the muscle in the controls and GLP-1 treated animals.

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1. INTRODUCTION

Glucose as the most stable simple sugar, serves as a precursor for a number of important cellular constituents such as glycogen, fat, amino acids and nucleic acids. Also as the major hexose circulating in a bloodstream, glucose serves as one of the most significant energy substrates for the cell (Roehrig, 1984). Living organisms have developed many efficient mechanisms for the conservation and regulation of fluxes of this essential metabolite. The task of maintaining stable blood glucose levels (i.e. glucose homeostasis) requires a balance between the glucose entry into and removal from the circulation. This balance is relatively stable in resting subjects in the postabsorptive state (steady state). Specific adjustments are required at times of massive glucose entry (e.g., a carbohydrate meal) or glucose removal (e.g., exercise), when a non-steady state occurs (Szabo & Szabo, 1986).

Glucose may enter the circulation via three major routes. The primary source is the environment, when glucose enters the body from the gastrointestinal tract during food absorption or less commonly, by the parenteral route such as intravenous nutrient administration. Another route of glucose entry is the liver which, in the course of glycogenolysis (i.e., the release of glucose originating from enzymatic breakdown of stored complex carbohydrates, mainly glycogen) and gluconeogenesis (i.e., the formation of glucose from noncarbohydrate precursors), supplies the body with the necessary amount of fuel. Finally the kidneys are capable of producing glucose from nonglucose precursors in the process of renal gluconeogenesis (Szabo & Szabo, 1986). The renal contribution to glucose production realized by this gluconeogenic pathway was recently estimated as 13–24 per cent

(Cherrington et al., 1994).

Glucose transport into the cell is essential for maintenance of glucose homeostasis. The major scheme for glucose entry into most of the cells is occurring by means of facilitated diffusion. In this process, specific integral membrane proteins passively transport glucose molecules down its concentration gradient (Kahn, 1994). A family of five facilitative mammalian glucose transporters (GLUT1–GLUT5) have been identified, the most characteristic feature of which is their specific tissue distribution (Bell et al., 1990). Two glucose transporters, namely GLUT1 and GLUT4, are expressed in highly insulin-sensitive tissues, such as muscle and adipose tissue. In particular GLUT4, expressed in skeletal muscle, heart and adipose cells (Thorens, et al., 1990; Burant, et al., 1991; Kahn, 1992) plays an important role in enhancing glucose transport in response to insulin in these tissues (Holman et al., 1990; Kahn, 1992).

The first step of glucose utilization upon its entry into the cell is phosphorylation to form glucose 6-phosphate. This process when occurring in the liver is catalysed by an enzyme of the group of glucokinases, glucose 6-phosphatase. In the nonhepatic tissues, other enzymes of the group of hexokinases seem to be involved. The process of glucose phosphorylation is completely irreversible, except in the liver cells, renal tubular epithelium and intestinal epithelial cells (Guyton, 1987 ; Gerich, 1993). The successive steps of chemical reactions during the ongoing process of glycolysis convert glucose into pyruvate with concomitant generation of molecules of ATP and NADH. This provides the cell with energy and carbon backbones for further biosyntheses. In an aerobic environment, glycolysis proceeds to the citric acid cycle and electron-transport chain, which together are responsible for harvesting most of the energy stored in the glucose molecule. If the oxygen supply is

insufficient, for example in actively contracting muscle, most of the pyruvate is converted into lactate. Both glycolytic products, pyruvate and lactate (anaerobic conditions) easily diffuse out of the cells through specifically permeable cell membranes and are carried to the liver (Stryer, 1988). Lactic acid can also readily diffuse into extracellular fluid and be taken up directly by less active, energy-deficient cells (Guyton, 1987). Notably, the red blood cells, gut and liver appear to be important sources of newly-synthesized lactate, however their particular contributions to the overall production of this metabolite are still unknown (Moore et al., 1991).

Gluconeogenesis is essentially the reversal of glycolysis and occurs in two organs : the liver and kidneys. In this process the major noncarbohydrate precursors are pyruvate, lactate, glycerol, alanine as well as other (glucogenic) amino acids. Gluconeogenesis from lactate represents the Cori cycle. During this process, as described by Cori (1931) glucose breakdown with formation of lactate occurs in peripheral tissues. Subsequently, reconversion of these precursors into glucose occurs in the liver. The fraction of gluconeogenesis occurring via the Cori cycle in normal subjects was estimated initially to range from 12 to 20 per cent (Reichard et al., 1963). More recent findings indicate values of 20 to 30 per cent (Felig, 1973) and 40 per cent (Randle et al., 1988). As it was mentioned earlier, the kidney cortex shares with the liver the capacity for gluconeogenesis from a number of noncarbohydrate precursors.

In addition to its role as a source of energy and a building block for biosynthetic processes in the cells, glucose can be stored in the form of a large, branched polymer molecule – glycogen. Particularly, from all cells capable of storing glycogen, the liver cells are able to accumulate up to 5 to 8 per cent of their weight as glycogen, whereas muscle cells only store

up to 1 to 3 per cent. The liver's glycogen serves as an important reserve of energy, which can be readily released in the form of glucose molecules into the bloodstream. In contrast, the muscle cells do not have the capacity to contribute its glucose into the circulation. Instead, muscle glycogen is broken down to provide energy for its work. Glycogen stored in adipose tissue liberates glucose molecules, which in turn reesterify fatty acids, entering the adipose tissue from the blood. Generated fat appears to be the source of two and a quarter times as many usable calories as the same amount of glycogen and serves as an important form of energy storage which can be released in critical conditions of fuel deficiency (Guyton, 1987 ; Stryer, 1988). In contrast, in the presence of abundant supply of exogenous glucose, hepatic glycogen stores can be repleted via two pathways : direct and indirect (gluconeogenic). In the first, phosphorylation of glucose and its incorporation into glycogen occurs. The gluconeogenic pathway involves initial systemic metabolism of glucose to gluconeogenic precursors (mainly lactate and alanine) and thence, their resynthesis to glucose, which upon its phosphorylation gets incorporated into the liver's glycogen (Radziuk, 1982, 1989 a, b).

The process of uptake, production and storage of glucose and gluconeogenic metabolites relies primarily on the coordination exerted by two major glucoregulatory hormones: insulin and glucagon, products of the activity of the endocrine pancreas. The major components responsible for release of these glucoregulatory hormones are the islets of Langerhans, clusters of endocrine tissue scattered throughout the exocrine pancreas (Falkmer & Ostberg, 1977). The islets themselves, accounting for 1 to 2 per cent of the adult pancreatic mass, consist of over 1 million cells, typically collected in aggregations of a few to several thousand cells (Weir & Bonner-Weir, 1990; Bonner-Weir, 1991). There are four major endocrine cell types in mammalian islets: the insulin-producing B-cell, the glucagon-pro-

ducing A-cell, the somatostatin-producing D-cell, and the pancreatic polypeptide-producing PP-cell. With an application of sensitive immunostaining techniques, numerous other peptides and hormones (e.g., vasoactive intestinal polypeptide (VIP), substance P (SP), gastrin) have been localized in the islet cells. The components of the islets of Langerhans are organized into three-dimensional architecture, which facilitates various levels of interactions and regulatory processes occurring via blood, interstitium and neuronal pathways (Bonner-Weir, 1991).

The human insulin molecule has a molecular weight of approximately 6,000 daltons and contains 51 amino acids arranged as two polypeptide chains. The A chain contains 21 amino acids and is linked to the B chain by two disulphide bridges. Insulin is synthesized by way of a complex pathway, involving several cellular organelles and at least two intermediates—preproinsulin and proinsulin. After the proteolytic cleavage of proinsulin to insulin, part of the proinsulin connecting peptide consisting of 31 amino acids, remains intact as the "C-peptide". The proteolytic cleavage of proinsulin to insulin within the Golgi lamellae and immature B-granules, results in the retention of equimolar quanta of C-peptide and insulin in the mature secretory granules. Therefore, the B-cell secretory products include equimolar amounts of insulin and C-peptide (approximately 95 per cent) and minute amounts of proinsulin and intermediate cleavage forms (remaining 5 per cent). The secretion of C-peptide along with insulin on an equimolar basis appears to be a convenient method of disposal for the synthetic by-product, since apparently it does not display an acute biological activity (Montague, 1983).

The secretory response of the B-cell to glucose is rapid and biphasic in normal individuals. The specific pattern of insulin release involves an almost instantaneous spike-like first

phase, maintained for a period of about 10 minutes, followed by a nadir and a slowly rising second phase (Pfeifer & Broadstone, 1991). The molecular basis of this phenomenon probably reflects the presence of two pools of secretory granules in the B-cell. Whereas the first phase is entirely dependent upon liberation of stored molecules of insulin, the second phase depends on insulin previously stored, as well as of the newly synthesized (Hedeskov, 1980). Concomitantly, as shown by Pfeifer et al. (1981), the first phase of insulin secretion is independent of steady state endogenous glucose levels, whereas the second phase of insulin release appears to be sensitive to pre-stimulation by glucose present in the bloodstream.

Insulin is a potent hormone affecting metabolism and function of most tissues of the body, particularly targeting in the liver, muscle and adipose tissue. It affects the metabolism of carbohydrates, lipids and amino acids by altering the transport of molecules and by influencing the activities of enzymes involved in this metabolism.

The action of insulin on carbohydrate metabolism includes stimulation of the removal of glucose from blood and its utilization by insulin-sensitive tissues (Wall et al., 1957). The latter is realized by stimulating a specific glucose transport system (Wardzala & Jernrenand, 1981) as well as stimulating glucose metabolism (Mandarino et al., 1987). Insulin promotes glucose storage and utilization by three energetically important tissues: muscle, fat (adipose tissue) and liver. The storage of glucose by muscle and liver is accomplished by activation of glycogen synthase, which is the enzyme controlling the process of glycogenesis. In addition insulin inhibits glycogen breakdown (glycogenolysis) by affecting the activity of the key enzymes from the group of phosphorylases. Insulin also promotes glucose oxidation or lipogenesis, particularly when glycogen stores become saturated. The stimulative effect of insulin on glucose transport in muscle and adipose cells results from a cascade of cellular events. Insulin binds to its receptor, evoking a series of intracellular signals. As a result of

this binding, exocytic-like translocation of GLUT4 glucose transporters towards the cellular membrane occurs, and enables their exposure to the extracellular milieu and subsequent activation (Kahn, 1992).

Generally, glucagon is an insulin antagonist. In normal circumstances the relative concentrations of these two hormones determine whether fuel needs to be mobilized or stored. The major site of glucagon action is the liver, where at physiological concentrations it promotes rapid mobilization of glucose stored in the form of glycogen in the process of glycogenolysis. At the same time, this A-cell pancreatic hormone aids the production of glucose from noncarbohydrate precursors (gluconeogenesis) and production of ketone bodies from fatty acids. The metabolic effects of glucagon at the level of adipose tissue contribute to mobilization of fatty acids and glycerol, stored as triglycerides in a process of lipolysis. The fatty acids after their release are used as a source of energy for the peripheral tissue and simultaneously provide a substrate for hepatic ketogenesis. In muscle and liver, glucagon also contributes to the process of protein breakdown, (proteolysis) increasing the release of amino acids, many of which serve as a substrate in hepatic gluconeogenesis (Montague, 1983).

The liver is the first target organ for the action of the pancreatic regulatory hormones. Anatomically it is positioned to function as a potential filter of newly absorbed nutrients. The liver thus plays a key role in processes of glucoregulation. A large share of the circulation, which flows through the stomach, intestines, spleen and pancreas finally routes into the portal venous system, and then to the liver. The portal vein as well as the hepatic artery, after entering the liver, branch into a network of hepatic sinuses, which establish close contact with the liver parenchymal cells, and finally converge into hepatic veins draining into the

inferior vena cava (Douglass et al., 1950 ; Guyton, 1987).

With regard to maintenance of glucose homeostasis in blood, it has long been suspected that hepatic uptake of glucose during its absorption from the gut may be favoured as a consequence of perfusion of the liver cells with portal blood containing relatively high concentrations of nutrients. The importance of glucose load reaching the liver as the major determinant of glucose uptake and deposition by this organ has been recognized already in 1878 by physiologist Claude Bernard (Bernard, 1878). Early experiments carried out in vitro systems confirmed this hypothesis: a dose–response relationship between hepatic glucose and ratio of its uptake have been clearly drawn (Cahill et al., 1958; Bucolo et al., 1974; Davidson, 1981). Initial experiments in dogs (Soskin & Levine, 1952) supported the concept of a direct link between glucose levels and hepatic function, indicating that hyperglycemia per se was responsible for the transformation of the liver from a glucose–producing to a glucose–assimilating organ. However, in case of in vivo experiments the factor of pancreatic response to elevated glucose was not controlled, therefore the observed effects could not be differentiated from those resulting from endocrine influences. Concomitant studies were aimed at elimination of the hormonal factor, which possibly affected hepatic glucose kinetics. Liljenquist and colleagues (1979) in their study examined the effect of induced hyperglycemia and combined acute suppression of insulin and glucagon by the use of another pancreatic hormone, somatostatin. The results of the study confirmed that hyperglycemia displayed the ability to inhibit hepatic glucose production in the absence of a rise in insulin, but at the same time failed to induce its splanchnic uptake.

In the experiments cited above, hyperglycemia was maintained by infusion of glucose solely into a peripheral vein. Results from a number of studies suggested, that glucose uptake

by the liver is influenced by the route of glucose delivery. An increase in hepatic uptake of glucose following oral glucose loading as compared with intravenous administration has been reported by many authors. When an oral glucose load was added to a peripheral infusion in man, DeFronzo et al. (1978) reported a sixfold increase in net splanchnic glucose uptake. Working on a dog model, Ishida et al. (1983) found that the increase in uptake of glucose by the liver during absorption of an oral glucose load could be mimicked by infusion of glucose directly into the portal vein. The results also demonstrated increased glucose uptake when infused intraportally ($65\pm 7\%$), as compared to the ratio taken up by the liver when infused peripherally ($23\pm 5\%$). Studies by Adkins et al. (1984) and more recently by Myers et al. (1991) have demonstrated that in dogs intraportal glucose delivery increased its hepatic uptake by a factor of almost three. A novel hypothesis, originally proposed by DeFronzo et al. (1978), suggested that a "gut factor", a hormone or unidentified substance of gastrointestinal origin, may be responsible for the enhancement of splanchnic glucose uptake after its oral administration gained therefore broad experimental evidence.

Approximate ratio of glucose uptake by the liver was also equivocal; various laboratories estimated that the liver could take up 40 to 65 per cent of the absorbed glucose (Perley & Kipnis, 1967; Jackson et al., 1973; Felig et al., 1975). Against this background it was found in studies with administration of labelled glucose in dogs, that the major portion of ingested glucose appears peripherally (Steele et al., 1968). The assessment of the ratio of glucose uptake (and therefore its extraction) by the liver as a measure of improved tolerance to oral versus intravenous administration has been carried out by Radziuk et al. (1978 a). As result of this study, a value of approximately 8 per cent was obtained and this seemed to be independent of the route of glucose entry. Therefore, the role of the periphery in mediating the improved tolerance to oral glucose was investigated. A concomitant study applied in human

subjects, revealed about 50 per cent greater rate of glucose uptake (and therefore its extraction) by the forearm muscle during oral loading as compared to intravenous (Radziuk & Inculet, 1983). This increase correlated well with the plasma insulin concentrations, which drastically rose after glucose ingestion. The role of insulin in mediating the increased peripheral glucose tolerance was therefore suggested. Using method of concurrent intravenous infusions of glucose and insulin, it has been demonstrated that tolerance to glucose administered by this route matched quite closely to the response to oral load (Radziuk, 1983). The result of this comparative study again confirmed supposition, that insulin could mediate improved glucose tolerance at the site of the peripheral tissue. Since in physiological circumstances such elevations in insulin secretion occur following ingestion of a meal (glucose), a factor of intestinal origin stimulating insulin response during glucose absorption has been also proposed by this author (Radziuk, 1987).

In fact, the participation of a hypothetical " gut factor " in mediating the tolerance to glucose loads entering by different routes has been earlier documented in studies conducted by Elrick and colleagues (1964). These investigators were able to demonstrate that oral glucose evoked a significantly greater and more sustained insulin response , as compared to administered intravenously. The role of supposedly intestinal factors contributing to the augmentation of insulin secretion during an oral glucose challenge was confirmed also by McIntyre and co-workers (1965).

The term " incretin " , originally introduced by Zunz and LaBarre (1929) gained experimental evidence, therefore an attempt to identify humoral activity of the gut that enhances the endocrine secretion of the pancreas (i.e., insulin) was initiated. The potential of oral glucose to release more insulin than intravenous infusion, despite a smaller increase of the

blood glucose has been described as an "incretin effect", the term originally coined by Jensen (Jensen et al., 1976). Another important term frequently used to describe this glucose-dependent stimulation of insulin release by the gut was the "entero-insular axis", a term originally introduced by Unger and Eisentraut (1969). These investigators also confirmed that the magnitude and pattern of pancreatic islets' secretory response does not correspond entirely to the concentration of the nutrients in circulation. Instead, elicited hormonal response is compatible with the existence of an entero-insular axis. It was found later that this additional stimulation was glucose-dependent, thus precluding the development of significant hypoglycemia in response to the higher insulin levels. The anatomical positioning of the cells releasing the gastrointestinal hormones i.e. exposed to the luminal digestive products and in close proximity to the nutrient absorptive sites, was suggestive of the involvement of such a gut factor in functioning of this axis. Historically, the evidence supporting the role of gastrointestinal hormones in the entero-insular axis was initially equivocal. Insufficient purity of the preparations tested, and the pharmacological doses necessary to elicit responses, rendered the data somewhat incompatible with known physiology.

The first hormone to be investigated for its incretin activity was secretin. It was demonstrated in human subjects that injection of a crude preparation of the gut mucosal extract with biological activity similar to that of secretin significantly increased the disappearance rate (i.e. disposal) of intravenously administered glucose. Elevations in insulin levels were also reported (Dupre, 1964). These results were confirmed again by the laboratory of the same author some time later (Dupre et al., 1966). Such observations enhanced further studies aiming in determination of glucose-insulin interaction upon response to the administration of the gut mucosal extracts. In a latter study in man, Lerner and Porte (1972) compared the insulin response to repeated large doses of secretin with the acute response to glu-

cose injection. They noticed decreasing insulin release upon repeated secretin stimulation, whereas insulin levels after successive glucose challenges increased. Lerner and Porte concluded from these observations that possibly functionally separated pools of insulin were stimulated by secretin and glucose. The final elucidation of the supposed incretin activity represented by secretin was revealed with improvements in radioimmunoassay techniques. These assays specified for detection of the secretin radioimmunoactivity have failed to demonstrate such an elevation following ingestion of a mixed meal (Bloom et al., 1975) or of oral glucose load (Bloom, 1974; Boden et al., 1975). The necessary criteria of glucose-dependent insulinotropic potency of secretin have not been satisfied, because of impurity of gut mucosal extracts utilized in the former studies.

Chronologically, the second hormone investigated for its insulinotropic potency in a hyperglycemic environment was gastrin. Studies conducted by Unger et al. (1967), and Dupre et al. (1969), demonstrated monophasic and transitory insulin response to injected gastrin and this effect seemed to be poorly correlated to the state of glycemia. The lack of glucagonotropic effect of gastrin preparations has been also demonstrated.

The hormone which for the first time exhibited both insulin and glucagon stimulatory properties was cholecystokinin-pancreozymin. The original study was performed by Unger et al. (1967). This result has been also confirmed by Buchanan et al. (1968). However, with improvement of the techniques of purification of obtained extracts, the earlier results has been questioned again. Thus studies examining the biological activity of gastrointestinal extracts containing various concentrations of pure CCK-PZ were carried out by Rabinovitch and Dupre (1972). The results showed that highly purified cholecystokinin was unable to enhance either insulin or glucagon secretion, whereas both effects were observed

with 10 per cent pure material. This observation suggested that crude extracts of CCK-PZ contained another insulin secretagogue.

Subsequently, gastric inhibitory polypeptide (GIP) was identified and its name arising from its role in inhibition of gastric acid secretion (Brown & Pederson, 1970) has been introduced by Brown et al. (1975). An alternative nomenclature, defining GIP as "glucose-dependent insulinotropic polypeptide" was also proposed, emphasizing the insulinotropic properties exhibited in the presence of glucose (Dupre et al., 1973; Brown et al., 1975; Pederson & Brown 1976, 1978; Tseng et al., 1994). Experiments in man with intravenous infusion of GIP in doses simulating postprandial plasma levels, showed that the peptide enhanced the increase in plasma insulin during intravenous glucose infusion. GIP alone (i.e. without supplementary glucose load), however, had no effect on levels of insulin in blood when infused in similar doses (Dupre et al., 1973). Pederson and Brown (1978) investigated glucagon and insulin responses of the perfused rat pancreas to glucose in the absence and presence of GIP. A glucagonotropic effect of GIP was observed at low glucose concentrations. The potentiation of glucagon releases by GIP was not significant at glucose concentrations above 5.5 mmol, at which the insulinotropic effect begins to manifest itself. These experimental data clearly indicated that besides GIP, an additional insulinotropic agonist (agonists?) act in concert on the endocrine pancreas.

Although these observations were more difficult to reproduce in humans, two approaches have been designed. The subjects of the first series of experiments conducted by Salera et al. (1983), the group of young healthy men consumed three regular meals and maintained their routine daily activities. Diurnal profile of hormones: insulin, glucagon and GIP along with levels of metabolites (blood glucose, triglycerides) were determined. The observa-

tions demonstrated that while plasma insulin concentrations fluctuated in close relation to the timing of nutrient ingestion, the response of GIP remained sustained after breakfast, with minor increments following subsequent meals. These experiments demonstrated certain degree of correlation between GIP and corresponding insulin release, however existence of another incretin displaying insulinotropic activity was also suggested.

The second series of studies utilized the blocking properties of prostaglandins of the E-series on GIP-release after oral glucose load. A priori it was verified that this procedure did not influence carbohydrate absorption. Enprostil (Schwartz et al., 1988) and misoprostol (Ebert et al., 1987) are known inhibitors of the GIP response to oral glucose in humans. After administration of these agents, the plasma insulin response was only marginally affected, indicating that additional factors might substitute for GIP as incretins (Nauck et al., 1989). The sensitivity of GIP to changes in blood glucose levels, and furthermore, liberation of an appropriate glucoregulatory hormone provides a fine-tuning mechanism for maintaining normoglycemia, and at the same time, prevents the risk of development of hypoglycemia. Based on its actions described above, GIP indeed seemed to represent one of the most serious incretin candidates (Creutzfeldt, 1979, Creutzfeldt et al., 1983; Ebert, 1990). It is released following carbohydrate ingestion, it amplifies the insulinotropic effect of glucose on the islets and is a safeguard against hypoglycemia (Elahi et al., 1979).

In the following years, however, evidence has accumulated which indicated that under certain conditions the incretin effect could not be explained solely on the basis of the action of GIP. The first observation came from experiments in rats, which were pretreated with GIP antiserum. Ebert and Creutzfeldt (1982) reported that after an intraduodenal glucose load and neutralization of endogenous GIP with specific antiserum as would be predicted, the early insulin response was blunted. However, the late insulin response was not altered,

despite complete binding of endogenous GIP to its antibodies. This observation would suggest that the incretin effect of GIP appears to be the strongest immediately after the glucose load. A subsequent series of experiments, led by the same group of investigators, examined the insulinotropic effect of crude gut extracts in rats. These preparations were capable of stimulating insulin release, but those with GIP removed by immunoprecipitation still exhibited more than 40 per cent of the insulinotropic activity (Ebert & Creutzfeldt, 1982).

The hypothesis suggesting the existence of other insulinotropic substances within the gastrointestinal tract was soon confirmed with the finding that many of the members of the glucagon family of peptides also stimulate insulin secretion from the pancreas.

The mammalian glucagon precursor (proglucagon) is a 180-amino acid protein, which was determined from the nucleotide sequence of the glucagon gene in hamster (Bell et al., 1983 b) and human (Bell et al., 1983 a) pancreatic islets. In both sites it showed an identical organization of sequences, determining five major functional regions: a signal peptide, an amino-terminal peptide (glicentin-related pancreatic peptide, GRPP), glucagon, and two carboxy-terminal glucagon-like peptides (GLPs): GLP-1 and GLP-2 (Bell et al., 1983 a). However, the gene nucleotide sequences did not provide information about the post-translational processing of the peptide precursor, which appeared to be different in pancreatic and ileal locations.

It was shown in the porcine and human pancreas that the main end-products of this processing are a glicentin-related pancreatic peptide (proglucagon 1-30, GRPP), glucagon itself (proglucagon 33-61) and a large amino acid composition (proglucagon 64-159) which contains the sequences of both GLP-1 and GLP-2 (Moody et al., 1981; Patzelt & Schiltz,

1984; Orskov et al., 1991; Orskov, 1992). The GLP-1 and GLP-2 molecules linked by an intervening sequence of a short spacer peptide, establish a compound unit called "major proglucagon fragment" (MPGF), which is not further processed at this site (Patzelt & Shiltz, 1984) and is released as a high molecular weight entity into the circulation (Orskov et al., 1991). Its biological importance is still unknown (Weir et al., 1989; Orskov et al., 1991; Orskov, 1992; Holst et al., 1994).

In the small intestine, the proglucagon is processed towards formation of glicentin (proglucagon 1-69) and two smaller molecules of GLP-1 (proglucagon 78-108) and GLP-2 (proglucagon 126-159), which as judged by gel filtration analysis seem to be secreted as separate peptides (Orskov et al., 1986; Buhl et al., 1988). The sequences of the glucagon-like peptides showed remarkable homology with glucagon: in the case of GLP-1 there is analogy in 14 amino acid residues, and in GLP-2 molecules, there are 11 amino acids in identical positions as glucagon (Schmidt et al., 1985; Orskov et al., 1986; Schjoldager et al., 1989; Goke et al., 1991). Many laboratories also reported a complete conservation of the amino acid sequences among various mammalian species, such as the rat, hamster, guinea pig, bovine, ox and also in man (Bell et al., 1983 a, b; Lopez et al., 1983; Heinrich et al., 1984; Seino et al., 1986; Holst et al., 1987; Orskov et al., 1989). This remarkably high degree of homology during the evolution suggests an important biological role for GLP-1 (Komatsu et al., 1989; Goke et al., 1991).

Two biologically active forms of GLP-1 are generated after N-terminal deletion of six amino acids, the GLP-1-(7-37) and its carboxy-amidated form, the GLP-1-(7-36) amide, also known as a truncated GLP-1 or t-GLP-1 (Orskov et al., 1986, 1988; Kreymann et al., 1988). Since, as shown by Holst (1983) and Adelhorst et al. (1994), the N-terminal part of the glucagon molecule is essential for its biological activity, and the truncated form

of GLP-1 displays stronger biological activity than the nonamidated form, the synthetic amidated GLP-1 is commonly used in experimental procedures.

The presence of specific GLP-1 receptors has been shown in several studies. Orskov and Nielsen (1988), and Goke and Conlon (1988) have identified binding sites for GLP-1 in rat insulinoma cell lines RIN 5AH and RIN m5F respectively, which are known to have properties similar to normal B-cells (Bhathena et al., 1982; Praz et al., 1983). Also the mouse insulinoma β TC-1 cells (Gefel et al., 1990; Fehman & Habener, 1991 b) and hamster insulinoma HIT-T15 cells (Fehmann & Habener, 1991 a) have been shown to demonstrate the presence of GLP-1 receptors. In addition to B-cells, somatostatin producing D-cells, also displayed the ability to bind GLP-1 (Goke et al., 1989 a; Fehmann & Habener, 1991 b). Evidence that in both cases binding of GLP-1 resulted in dose-dependent increases of cAMP levels (Drucker et al., 1987; Goke et al., 1989 a; Gefel et al., 1990; Fehmann et al., 1992) led to the assumption that effects of the peptide on B- and D-cells are coupled to adenylate cyclase system. After initial cell-surface binding, the hormone appears to be internalized by means of receptor-mediated endocytosis, and it is likely that this internalization is related to the action of GLP-1 on B-cells (Goke et al., 1989 b). These investigators demonstrated also, that intracellular cleavage of the hormone is facilitated by lysosomal degradation, since it was decreased in the presence of chloroquine, a potent inhibitor of intracellular peptide degradation (Lie & Schofield, 1973). It requires further examination whether GLP-1 or its degradation product may have any effect on intracellular metabolism.

The potential to displace GLP-1 from its binding sites by GLP-2, GIP, VIP, secretin, oxyntomodulin – peptides of the same family of glucagon – and glucagon itself was tested

(Schmidler et al., 1994). The results showed that glucagon, GLP-2 and oxyntomodulin, products of the proglucagon gene were three to four orders of magnitude less potent displacers of GLP-1 from its apparently high-affinity receptors, whereas GIP, VIP and secretin were ineffective.

GLP-1 was found to be secreted in response to meals : levels of the circulating peptide were measured in healthy humans by Kreyman et al. (1987). While fasting plasma concentrations of this hormone (mean \pm SEM) were determined as 15 ± 6 pmol/l, within 45 min following oral glucose drink (200 ml water containing 75 g glucose) the levels of GLP-1 increased to 41 ± 4 pmol/l.

Glucagon-like peptide-1(7-36) amide has been shown to be a potent insulin secretagogue. In the isolated perfused rat, porcine and canine pancreas, using models in which the vascular architecture was maintained, GLP-1 released insulin at approximately physiological concentrations (Schmidt et al., 1985; Holst et al., 1987; Mojsov et al., 1987; Goke et al., 1989a; Komatsu et al., 1989; Kawai et al., 1989; Weir et al., 1989; Orskov et al., 1993). Stimulation of insulin secretion by GLP-1 has also been shown in several cultured insulinoma cell lines and isolated islets (Goke et al., 1989 a; D'Alessio et al., 1989; Gefel et al., 1990; Fehman & Habener, 1991 a, b, 1992; Nathan et al., 1992; Montrose-Rafizadeh et al., 1994). GLP-1 exerts its diverse insulinotropic effects on B-cells via the pathway of stimulation of cyclic AMP formation, which is linked to insulin biosynthesis, expressed as a rise in insulin mRNA transcripts as well as achieved insulin levels (Drucker et al., 1987; Fehmann & Habener, 1992; Fehmann et al., 1992), and finally to the release of insulin molecules into the circulation (Kreyman, 1987; Komatsu et al., 1989; Namba et al., 1990).

What supports the hypothesis that GLP-1 is a strong incretin candidate is that, along with its insulinotropic action, a significant and self-limiting dependence on plasma levels of glucose is found. In B-cells of the perfused rat pancreas Komatsu and colleagues (1989) found that the potency of GLP-1 was greater in the presence of high glucose concentrations (11.2 mmol) than in the presence of low glucose concentrations (2.8 mmol). Also in the rat perfused pancreas Weir and his associates (1989) demonstrated that increases in ambient glucose within the physiological range, resulted in the ability of GLP-1 to stimulate insulin secretion. Whereas no detectable insulinotropic response was observed at the concentration of glucose of 2.8 mmol, the augmented release was documented at glucose concentrations of 6.6 and 16.7 mmol. Similar results, confirming the hypothesis of the insulinotropic potency of GLP-1 enhanced by additional stimulation exerted by elevated glucose levels, have been reported by D'Alessio et al. (1989), and Montrose-Rafizadeh et al. (1994). A study in humans was performed by Kreymann et al. (1987), where GLP-1 (7-36) amide was infused under fasting conditions at a rate which mimicked its postprandial levels (0.5 pmol/kg/min). This procedure demonstrated a small glucose lowering and insulin elevating effect. However, when an intravenous infusion of glucose (1g/min) was added, GLP-1 greatly enhanced insulin release and significantly reduced plasma glucose concentrations, as compared with a group of controls where saline was infused in place of GLP-1. The pattern and quantity of insulin release after GLP-1 stimulation have been also assessed. Weir and co-workers (1989) demonstrated in the isolated perfused rat pancreas that the pattern of insulin response was biphasic. The first phase lasting for a few minutes, was followed by a more prolonged period of sustained insulin secretion. This characteristic secretory response of insulin was similar to that seen during stimulation with other secretagogues, such as glucagon, somatostatin, and pancreatic polypeptide.

The molecular basis of the regulation which GLP-1 exerts on the B-cell signalling system was investigated by Holz and colleagues (1993) in isolated B-cells from rat islets maintained in a short-term cell culture. Findings of this study indicated that GLP-1 synergizes with glucose on a pathway of inhibition of the activity of ATP-sensitive channels (I_{KATP}). As a consequence, B-cells depolarize generating an action potential that results in insulin secretion. The process displays an absolute requirement for glucose. In addition to enhancing the efficiency of individual B-cells in this way, GLP-1 may also recruit more B-cells to secrete insulin (Montrose-Rafizadeh et al., 1994).

Another significant, but still not entirely elucidated action of GLP-1 is related to its inhibitory influence on another pancreatic hormone, glucagon. The study carried out in man by Kreymann et al. (1987) showed that intravenous infusion of GLP-1 (7-36) amide along with glucose led to significant glucagon restraint. Weir and co-workers (1989) however did not find detectable glucagonostatic effect of GLP-1 (7-37) in isolated perfused rat pancreas, whereas Komatsu et al. (1989) reported that GLP-1 amide was able to exert glucagonostatic action in a similar environment. In addition to the isolated perfused rat pancreas (Kawai et al., 1989; Suzuki et al., 1989; Komatsu et al., 1989), GLP-1 amide has been shown to inhibit glucagon secretion in the systems of isolated perfused pancreases, namely in the pig (Orskov et al., 1988) and dog (Kawai et al., 1989). However in systems in which the architecture of the islets was destroyed (e.g., islet cell monolayer cultures), no glucagonostatic effect of GLP-1 was shown (D'Alessio et al., 1989). Moreover, in the pancreatic A-cell lines producing glucagon (INR 1G9), binding sites for GLP-1 were not observed and therefore the possibility of the peptide to influence secretion of glucagon was excluded (Fehmann & Habener, 1991 b). This finding led to suggestion that the eventual effect of GLP-1 exerted on glucagon-secreting cells, which has been observed in some of the ex-

periments, may be mediated by indirect pathways, such as the ones involving action of insulin and somatostatin (D'Alessio et al., 1989; Weir & Bonner-Weir, 1990; Fehmann & Habener, 1991 b; Orskov, 1992). This hypothesis was supported by the observation documented in a rat model by Samols et al. (1988), that arterial blood acting within the islets as a local portal system, flows first to the centrally located B-cells (core of the islets), and then to the peripherally located (mantle of the islets) A- and D-cells, which are therefore exposed to the highest insulin concentration that exists in the circulation. The B→A→D order of cellular perfusion is therefore responsible for the regulation of the net secretion of the pancreatic hormones; the B-cell which is the primary glucose sensor inhibits the A-cell and also the D-cell. Noteworthy, studies in the dog (Stagner et al., 1988) and the rhesus monkey (Samols et al., 1989) suggest microcirculation similar to the rat order of islets. There is also evidence documented (Taborsky, 1983; Samols & Stagner, 1990) that somatostatin can possibly suppress the glucagon secretory response by means of "paracrine mechanisms". The hypothesis of paracrine regulation, proposed by Unger and Orci (1977), suggests that diffusion of peptides and their mutual regulation is being realized throughout intestinal components, especially in relation to A- and D-cells, which are anatomically adjacent. The influence of GLP-1 on another pancreatic hormone somatostatin, has been studied by several groups of investigators.

Fehmann and Habener (1991 b) identified binding sites for GLP-1 on somatostatin-producing D-cells. Binding of the peptide to these receptors resulted in increased generation of cAMP and, in consequence, liberation of somatostatin molecules. Similar stimulatory effects of GLP-1 on somatostatin secretion were also reported in a perfused pig pancreas (Orskov et al., 1988) and dog pancreas (Kawai et al., 1989). Since somatostatin is a known mediator of the suppression of glucagon during hyperglycemia (Gerich et al., 1974), it seems reasonable to suggest that the glucagonostatic effect of GLP-1 may be indirectly me-

diated by this hormone (D'Alessio et al., 1989; Weir & Bonner-Weir, 1990; Fehmann & Habener, 1991 b; Orskov et al., 1992).

Finally, direct actions of GLP-1 on other, nonendocrine tissues have been suggested. Determination of the tissue distribution of GLP-1 receptor mRNA performed by Thorens (1992) detected the presence of GLP-1 binding sites not only in the pancreatic islets, but also in stomach and lung membrane preparations. No GLP-1 receptor however could be detected in brain, liver, thymus, muscle, intestine and colon. This report has been recently reexamined by Egan et al. (1994). Their findings have shown the presence of GLP-1 receptor mRNA in a range of rat tissues, including leg muscle, liver, heart, kidney, intestine, brain, epididymal fat, as well as cultured 3T3-L1 adipocytes, but not in abdominal fat or rectus muscle. With regard to the differences in insulin responsiveness between various locations of fat tissue as well as considering the factor of fiber type and capillary density in muscle tissue (James et al., 1985, 1986), there still remains under speculation the possibility of differential insulinotropic effects exerted by glucagon-like peptide-1.

In addition to GLP-1-stimulated insulin release in healthy human subjects, the increased glucose effectiveness and insulin-independent glucose deposition, without significant change in insulin sensitivity have been reported (D'Alessio et al., 1994). Such an insulin-independent glucose disposition may play a role in glucose disposal in the postabsorptive period as well as in the basal state, and is realized primarily by the central nervous system (Service, 1995). The increased glucose effectiveness gives a global measure of the effects of changes in glucose concentration on glucose tolerance, and includes both insulin-dependent and insulin-independent mechanisms (D'Alessio et al., 1994). This phenomenon may perhaps explain the fact observed by Gutniak and colleagues (1992) of increased glucose uptake in diabetic patients treated with GLP-1 infusions.

All these observations support the notion that GLP-1 may exert diverse effects on the endocrine pancreas, as well as peripheral tissue levels. These processes, mediated via insulin-dependent and insulin-independent mechanisms, contribute significantly to regulation and maintenance of the glucose homeostasis.

The concept of hormonal factors involved in the pathogenesis of metabolic diseases, such as non-insulin-dependent diabetes mellitus (NIDDM, type-2 diabetes) has been investigated in the context of "insulin resistance". This term describes the inability of insulin to lower plasma glucose effectively, and therefore, incapacity of the B-cell to compensate sufficiently for hyperglycemia (Himmsworth & Kerr, 1942). Non-insulin-dependent diabetes mellitus is a disorder of glucose homeostasis, characterized by both fasting and postprandial hyperglycemia. In the pathogenesis of this state a number of factors responsible for regulation of blood glucose levels may be involved (Weir & Leahy, 1994). Islet cell interactions in diabetes may undergo a number of quantitative changes, such as B-cell deficiency or reduced ability to secrete insulin; as well as qualitative changes reflecting derangements in control of their secretory activity (Weir & Bonner-Weir, 1990). A leading hypothesis suggests that these changes in B-cell function result from deleterious effects of chronic hyperglycemia, which directly affects the B-cell sensitivity (Weir et al., 1986). In healthy subjects, the fasting (postabsorptive) levels of plasma glucose are maintained by an equilibrium between two processes: hepatic glucose production (HGP) and glucose uptake by peripheral tissue. Regulation of HGP is provided primarily by the combined effects of hyperglycemia, insulin and glucagon (DeFronzo & Ferranini, 1987; Cherrington et al., 1987). As a result of impaired insulin secretion and resistance of the liver to this hormone (Campbell et al., 1988), this major glucose-producing organ is being promoted to enhanced

glucose production, what in consequence leads to the increases in plasma glucose levels (Beck-Nielsen et al., 1992; Consoli, 1992).

Secretion of another important gluconeogenic regulator glucagon, also appears to be abnormal in NIDDM. Its plasma levels have been found to be elevated in type-2 diabetes mellitus during the postabsorptive states (Unger et al., 1970; Felig et al., 1976; Claus et al., 1983). This pathological hypersecretion of glucagon profoundly enhances hepatic glucose production in NIDDM (Consoli, 1992). Neither of the usual controlling factors is able to regulate this elevated glucagon secretion; on one side A-cell responsiveness to hyper- and hypoglycemia is blunted (Bolli et al., 1984), on the other side insulin does not seem to compensate sufficiently for glucagon hypersecretion (Unger & Orci, 1994).

Elevated, as compared to normal, glycemic excursions seen in type-2 diabetic patients resulted from a failure of the inhibition of hepatic glucose production in combination with reduced glucose uptake by the muscle (Firth et al., 1986; Ferranini et al., 1988). This situation was a direct consequence of increased insulin resistance at the site of peripheral tissue (DeFronzo et al., 1982; Bogardus et al., 1984).

The hypothesis that lack of a duodenal factor which may enhance glucose disposal is responsible for some forms of diabetes, originally formulated by Moore et al. (1906), gained recently renewed interest. Since in type-2 diabetes mellitus the "incretin effect" is reduced or lost (Nauck et al., 1986; Tronier et al., 1985), a number of studies focused on a hypothetical intestinal factor responsible for enhanced insulin secretion following an oral glucose challenge. The first incretin to be examined, gastric inhibitory polypeptide (GIP), failed to show significant insulinotropic effect in diabetic patients (Nauck et al., 1986, 1993). Since GLP-1 was proposed as another intestinal factor with well documented incretin properties, its use in treatment of NIDDM was therefore postulated (Kreymann et al., 1987; Gutniak et al., 1992; Nathan et al., 1992; Orskov et al., 1993; Nauck et al., 1993). It was

shown in experiments performed by Gutniak (1992) on patients with NIDDM, that the infusion of GLP-1 enhanced endogenous insulin secretion, improved its sensitivity and decreased glucagon release. These effects together accounted for a decrease in the meal-related insulin requirements substantially, therefore demonstrating that administration of GLP-1 may be used as a possible treatment in this disorder. The property of normalizing plasma glucose concentrations in poorly-controlled type-2 diabetic patients has been well documented by Nauck et al. (1993). Although the actions of GLP-1 observed in this study were pharmacological rather than physiological (two- or even threefold higher than peak postprandial values), the insulinotropic and glucagonostatic action of the hormone was achieved and reversed immediately after approaching normal fasting values of glucose in blood, thus preventing the incidence of hypoglycemia. Holz and colleagues (1993) investigated the role of GLP-1 as a possible hormonal regulator of the B-cell-glucose signalling system, which appears to be impaired in NIDDM. Their findings indicated that GLP-1 indeed synergizes with glucose in order to inhibit the ATP-sensitive potassium channels (I_{KATP}), thereby allowing B-cells to depolarize and generate action potentials, events that are necessary prerequisites to glucose-stimulated insulin secretion. The likelihood for development of hypoglycemia during the treatment with intravenous infusion of GLP-1 was reduced since a rise in circulating insulin, accompanied by lowering blood glucose effects, were self-terminating processes. This extra margin of safety provided by treatment with GLP-1 offers advantages over sulphonylureas and insulin, use of which despite of the risk of development of the hypoglycemia, induces other pathological states, such as hyperinsulinemia or arteriosclerosis (Robertson et al., 1988; Scheen et al., 1989).

A number of effects exerted by GLP-1 has been hypothesized. In this study confirmation of insulinotropic and glucagonostatic (and therefore glucoregulatory) effects of GLP-1

was sought. Simultaneously we wished to determine whether GLP-1 can alter glucose uptake and, if so, whether this is direct or by means of mediators (such as insulin itself). Finally, we examined the glucoregulatory actions exerted by GLP-1 in a number of different tissues. To obtain normal physiological conditions, the experiments were performed in a large animal model (the pig). This facilitated the analysis of both metabolite and hormone kinetics continuously in time, concurrently with an assessment of their handling by individual tissues – all in response to exogenously induced changes in blood levels of GLP-1. Another part of the rationale for choosing the pig as a study animal model was the apparent similarity of its glucose metabolism with that of humans.

Thus in surgically prepared animals, following overnight fast and assessment of baseline parameters, an infusion of glucose was initiated to clamp its levels at a concentration where GLP-1 was expected to be effective (150 mg/dl). This was accomplished by constantly monitoring the glucose levels and adjusting glucose infusion rates. Two dose-rates of GLP-1 were then superimposed on this glucose infusion. Again, the rate of exogenous glucose administration was adjusted accordingly (in order to maintain the same level of hyperglycemia) and the overall response of glucose disposal and production by the GLP-1 infused was thus monitored. Simultaneously, arterial insulin, C-peptide, glucagon and GLP-1 concentrations were measured.

Secondly, in order to distinguish between different tissue effects of GLP-1, these effects were separately measured in the liver, the intestine and the muscle of the hindlimb. This was accomplished by determination of arterio-venous differences in hormone or metabolite concentration, combined with the measurement of blood (or plasma) flow through the considered tissue bed. The doses used (4 ng/kg/min and 8 ng/kg/min) were chosen, since similar infusion rates have been used in man to simulate the physiological excursions of GLP-1 (Kreymann et al., 1987).

Both, the systemic and tissue-specific changes occurring upon GLP-1 stimulation can be correlated to the levels of insulin and glucagon, in order to assess the degree to which each of these hormones mediates the effects of GLP-1. This work is therefore an attempt to dissect out the actions of GLP-1, the degree to which they take place, their targets and their possible mediators.

2. MATERIALS AND METHODS

2.1. Animals.

2.1.1. Animals and housing.

The study involved 20 pigs, of which 10 led to successful and complete studies. Their weight ranged from 16.8 to 37.3 kg (mean \pm SEM: 23.3 \pm 5.6 kg). Pigs were housed in Ottawa Civic Hospital vivarium in 12:12 hour light/dark cycle environment (light on at 0700 hours). Pigs were fed once per a day with a standard chow consisting of: carbohydrates (75%), protein (16%), fat (2.5%), fibre (6%) and essential minerals and vitamins. Tap water was available at libitum.

2.1.2. Surgery.

After an overnight fast (18 hours) pigs underwent surgery. They were anaesthetized with an intramuscular dose of atropine (0.05 mg/kg) and ketamine (11mg/kg). Saline (0.9 NaCl) was infused intravenously at a rate of 10 ml/kg/h. The rectal temperature was monitored regularly and maintained at a near-constant level by the use of a heating pad underneath the body. This is warmed with a saline infusion from a warm water bath. The pump respiratory rate was set at 12 breaths per minute. The electrocardiogram tracing was monitored constantly.

During surgery permanent blood sampling lines (Tygon, Northon Performance Plastics)

were inserted into the portal vein, hepatic vein, femoral vein and femoral artery.

The portal catheter was directed towards the superior mesenteric vein, with its tip laid below the juncture of the pancreatic vein into the portal vein. The hepatic venous cannula was advanced about 1–2 cm inside the common hepatic vein. Femoral arterial and venous sampling lines were placed in the opposite limbs. Three catheters were inserted into the left jugular vein for infusion purposes. All of the catheters were secured with a silk suture (2.0, Ethicon Inc., Canada) around the vessel. On the femoral artery, but in the opposite limb to that where arterial sampling takes place, the sensory unit of the ultrasound blood flowmeter was implanted. After checking for their patency, the catheters along with the output from the flow probe were threaded through a sterilized long needle and subcutaneously exteriorized on the pig's back, in the region of shoulders. Extensions with three-way stopcock (Mallinckrodt, Inc., Glens Falls, NY) were attached to the cannulas. The lines and the probe output were placed together in a pouch secured with an Elastoplast bandage (Smith & Nephew Inc., Lachine, Canada). Postoperatively, the catheters were flushed daily with a diluted heparin solution (100 U/ ml) in order to prevent thrombosis and maintain patency.

Experiments were done only on animals whose weight stabilized on resumption of eating (about 2 days postoperatively) and when this weight was maintained at the preoperative level for at least 1 week before studies. A successful recovery included good general health, normal appetite and stools.

2.2. Experimental procedures.

2.2.1. Objectives.

The following series of experiments were aiming at assessing the alterations in rates of glucose fluxes across metabolically important tissues (liver, gut, muscle) as well as the release of pancreatic hormones in response to hyperglycemia and increased blood levels of GLP-1. Both hyperglycemia and elevations in levels of circulating GLP-1 were produced by infusion.

2.2.2. Preparation of the infusates.

2.2.2.1. Indocyanine green (ICG).

50 mg of Indocyanine green (commercially available batches, Becton Dickinson and Co., Cockeysville, MD) was diluted to 60 ml with 1.6 ml of HSA (25% solution, Miles Inc., Etobicoke, Ont, Canada) and 0.9% NaCl (pH 5.7, Abbott Laboratories, Montreal, Canada). The solution was infused via a Harvard pump from the 60 ml syringe (Sherwood Medical, St.Louis, MO).

2.2.2.2. Glucagon-like peptide-1.

GLP-1 (7-36) amide (human) in the quantity of 2.356 mg per batch was obtained from BACHEM Bioscience Inc. (King of Prussia, PA, USA). To this amount 0.1 ml of 0.01N HCl was added, followed by 4.8 ml of saline and 0.1 ml of HSA. The GLP-1 was stored (at -85°C) in aliquots of approximately 30 µg per tube (approximately 63 µl). Before the ex-

periment, GLP-1 (30 µg/tube) was diluted with 1% HSA and saline to the volume of 60 ml and drawn up into the syringe used for infusion.

2.2.2.3. Glucose.

Glucose (20% Dextrose) was purchased from Baxter Corporation, Toronto, Ont., Canada, as a 500 ml bags and stored in room temperature until used.

2.2.3. Experimental protocol.

The pig, fasted for 18 hours was weighed and transported into the experimental room. During the 150 min of basal period, immediately after drawing baseline samples at the time=0, a constant (0.130 ml/min) infusion of indocyanine green (ICG) was initiated using a syringe infusion pump (model 22, Harvard Apparatus, South Natick, MA).

After dye equilibration at time=150 min, glucose (20% Dextrose, Baxter Corporation, Toronto, Ont. Canada) was infused at variable rates employing a pump (model 560 MEE, IVAC Corporation, San Diego, CA). Manual adjustment of the pump according to frequently sampled glucose levels served to maintain glycemia at the level of 150 mg/dl.

At time=240 min, while maintaining glucose concentrations at 150 mg/dl, two different protocols were instituted:

- (i) GLP-1 was infused at 4 ng/kg/min for 90 min, followed by a second step at 8 ng/kg/min for a further 90 min (number of animals used: n=5),
- (ii) Saline was infused at rates equivalent to the solution in (i) in corresponding control studies (number of animals used: n=5).

2.2.3.1. Sampling.

Arterial blood samples for glucose and hormone determinations were drawn at variable intervals, more frequently when rapid changes were expected. At the end of each period (glucose infusion alone, glucose infusion with either 4 or 8 ng/kg/min of GLP or saline in control studies), when steady state was most nearly attained, four samples were drawn at 15 min intervals from the portal, hepatic and femoral veins as well as a femoral artery. Micro-hematocrits were also done on the blood from these samples (Haemofuge, model 3612, Heraeus Sepatech GmbH, Germany), values ranged from minimum: $26.1 \pm 2.6\%$ to maximum: $34.4 \pm 3.3\%$, SEM: $30.3 \pm 5.9\%$. These hematocrits were used to monitor potential erythrocyte depletion as well as being used in calculations.

The sampling cannulas were flushed with heparinized saline and the withdrawn blood was replaced with red cells, after their separation from plasma and resuspension in normal saline.

2.2.3.2. Treatment of the blood samples.

Blood samples were withdrawn into dry sterile disposable plastic syringes (Becton Dickinson & Company, Franklin Lakes New Jersey) and transferred to the labelled heparinized glass tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ).

The samples withdrawn were divided as follows: 2.5 ml was for insulin and metabolite assays, and 1.5 ml for C-peptide, GLP-1 and glucagon determinations. To the latter tubes 150 μ l of Trasylol (Aprotinin, 10 000 K.I.U./ ml) was added. The vacutainers were centrifuged for 3 min at 4 000 r.p.m. (1 000 x g). The separated plasma was aspirated with a Pasteur pipette into another set of labelled tubes. Plasma samples were stored at -20°C until assayed.

When multiple samples were drawn, ICG was also assayed in arterial and hepatic venous samples, requiring an additional 1.5 ml of blood from each vessel. Plasma obtained from these samples was kept at room temperature until assay (within 24 hours).

2.2.4. Determination of blood (plasma) flow.

The measurements of blood and plasma flows were necessary in calculations of tissue metabolite and hormone balances.

2.2.4.1. Femoral vein blood flow.

The principle of differences in the transit time of the signal (ultrasound wave) travelling against and with the blood flow underlies the function of the Transonic Flowmeter (model T 101 D, Transonic Systems Inc., Ithaca, NY). The instrument consists of a bench – top electronic flow detection unit and perivascular flow–sensing unit. Whereas the surgically implanted probe is responsible for the generation and reception of the ultrasound signal, the electronic circuitry performs appropriate conversions so that flows are read from an analog scale. The measurements were done as soon as possible after the time of the corresponding blood sample.

2.2.4.2. Indocyanine green assay.

In order to obtain necessary data to calculate the liver plasma flow, the following procedure was employed. Immediately after termination of the experiment, the samples were treated with 1 µl of Arvin (Anticoagulant, 70 I.U / ml, Knoll Pharmaceutical, Canada), vortexed and left to stand overnight at the room temperature. On the next day the tubes containing

plasma specimens were spun in a microcentrifuge (model M 14–11, Jouan Inc., Cedex, France) at 10 000 r.p.m. (8 000 x g) for 30 min in order to remove the fibrinogen clot. In the meantime, a stock solution of the dye was prepared by a series of dilutions of the infusate in 1% HSA. The diluents were then transferred into appropriately labelled tubes containing baseline samples. On a basis of the standard curve thus obtained, the concentration of ICG in plasma samples was determined spectrophotometrically at 805 nm (Spectrophotometer, model U – 2 000, Hitachi Ltd., Tokyo, Japan)

2.2.4.3. Liver plasma flow.

This variable is calculated from the difference in the concentration of a dye, Indocyanine green (Cardio green, Fox green) between arterial and hepatic venous plasma. It was shown by Wheeler et al. (1958), that following intravenous injection, the dye is rapidly distributed in the circulation by its binding to plasma albumin. Caesar and co-workers (1961) have demonstrated lack of dye toxicity and the accuracy of this technique. A spectrophotometric method is employed to monitor the concentrations of dye in the plasma samples.

2.2.5. Determination of the concentrations of metabolite in plasma.

2.2.5.1. Glucose concentration.

Plasma glucose concentrations were measured employing the glucose oxidase method. In this process, glucose reacts under the influence of glucose oxidase (β -D-glucose : oxygen oxydoreductase) forming gluconic acid and hydrogen peroxide. The glucose analyzer (model 6517, Beckman Instruments, Inc., Fullerton, CA) measures the maximum rate of oxygen depletion which is directly proportional to the concentration of glucose (Kadish et

al., 1968). Approximately 10 µl of plasma is subjected to the analysis and the results are displayed digitally (mg/dl) within 20s after the sample application. This method of glucose concentration measurement was used in the experiments to aid in the maintenance of glucose levels at the preset values.

A modification of this technique is used in the YSI Glucose & Lactate Analyzer (model 2 300 STAT, Yellow Springs, OH). In this instrument free electrons are derived from the released hydrogen peroxide upon its reaction on a platinum electrode. The flow of these particles is linearly proportional to the H₂O₂ concentration and therefore to the concentration of glucose in sample. The volume of 25 µl of plasma was aspirated and the result of the analysis was electronically displayed and printed within 35 seconds.

2.2.5.2. Lactate concentration.

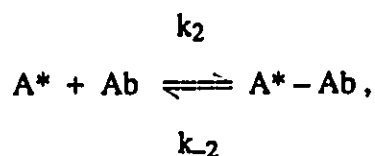
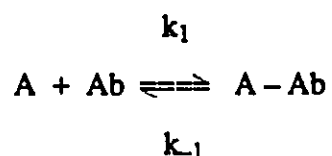
Plasma lactate concentrations were measured using the Yellow Springs Instruments Glucose & L-Lactate Analyzer (model 2 300 STAT). Analogous principle involving detection of the intensity of the flow of free electrons proportionally to the concentration of L-lactate in sample was utilized. In the case of determinations of lactate levels, the enzyme L-lactate oxidase was immobilized to catalyse this reaction.

2.2.6. Determination of the concentrations of hormone in plasma.

2.2.6.1. Basic principles of the radioimmunoassay technique.

The radioimmunoassay technique has been developed on the basis of the competitive binding phenomenon. According to Campfield (1983), the hormone reacts on a one-to-one basis with its highly specific antibody. Upon introduction of molecule of labelled hormone

of the same type, competition for the binding sites on a common antibody takes place. The reaction which occurs can be written as follows :



where : A is the antigen (hormone), A* is the labelled hormone, Ab is the specific antibody, and A - Ab and A* - Ab are the hormone - antibody complexes, natural and labelled respectively. The values k_1 and k_2 are the association constants , and k_{-1} and k_{-2} are dissociation constants. It is also assumed , that $k_1 = k_2$ and $k_{-1} = k_{-2}$. As a result of this competition, the quantity of each of the two forms of hormones that gets bound, is proportional to its concentration . The amount of A* - Ab complex can be quantitatively determined in a gamma counter. Using unlabelled hormone of known concentrations, a standard curve can be generated and the unknown concentration of hormone in plasma sample can be read from this curve.

A specific version of the radioimmunoassay technique, which involves use of the coated charcoal has been described by Herbert (1968). By its property of adsorbing molecules

of specific size, charcoal serves as a separating agent for two significantly different in weight and diameter molecular fractions of the solution . In order to render these adsorptive effects more specific for particles of certain sizes, coating material such as blood factors, plasma or dextrans can be applied to the surface of the charcoal. Such preparations may therefore be employed in the isolation of the bound and free fractions of hormones in plasma.

In all radioimmunoassays which were done here, plasma blanks (without antiserum addition), buffer blanks (again without antiserum addition) , standards (serial dilutions of hormone) and total counts (radiolabelled hormone only) were included for counting along with the sample being tested.

2.2.6.2. Insulin radioimmunoassay.

Plasma insulin was measured using a double antibody radioimmunoassay technique as described previously (Morgan & Lazarow, 1963). Monoiodinated insulin (^{125}I -human insulin, specific activity less than $5\mu\text{Ci}$ per vial) , first antibody (guinea pig anti-human-insulin, pancreatic specific), second antibody (goat anti-guinea pig IgG) and carrier (guinea pig normal carrier) were purchased from Linco Research, Inc., St. Louis, MO. A buffer solution (pH 7.4) was also prepared as follows: 0.57% di-sodium hydrogen orthophosphate (anhydrous), 0.1% sodium phosphate monobasic, 0.85% sodium chloride, 0.05% sodium azide, 1.04% EDTA, 1.0% BSA were dissolved in distilled water. Polyethylene glycol 8 000 (PEG 8 000) was added to part of the buffer in order to obtain a 3% solution. This reagent was then used for further dilutions of the carrier and second antibody. The tracer and first antibody were dissolved in 25 ml of the buffer without PEG. After 1/2 hour incubation at room temperature, the tracer mixture was aliquoted into 1.6 ml portions and stored at -20°C . The carrier along with first and second antibodies was stored in 5 ml of the diluent

at -20°C .

Briefly, the assay procedure involved addition to 100 μl of plasma or standard, 100 μl of each of: first antibody, tracer and the buffer. The content of the tubes was vortexed and allowed to incubate overnight at $+4^{\circ}\text{C}$. After 18 hours 100 μl of carrier and 100 μl of the second antibody were added. After vortexing, the mixture was left at $+4^{\circ}\text{C}$ for two hours. The tubes were then centrifuged (model PR 7 000 Centrifuge, International Equipment Company, Needham Hts, MA) at 2 000 r.p.m. (1 000 x g) for 15 min (temperature $+4^{\circ}\text{C}$). The supernatant was then decanted into a waste beaker and the remaining pellet was counted in Minaxi Gamma Counter (model 5 550 , Packard Instrument Company, Downers Grove, IL) for 5 min.

2.2.6.3. C-peptide radioimmunoassay.

In the procedure for determination of plasma concentrations of the connecting peptide, the Porcine C-Peptide Radioimmunoassay Kit (Linco Research, Inc., St. Louis , MO) was used. The specific activity of the tracer used (^{125}I -porcine C-peptide) was less than $3\mu\text{Ci}$ per vial.

The method required addition of 100 μl of porcine C-peptide antibody to 100 μl of sample or standard. The mixture was vortexed and allowed to incubate overnight at $+4^{\circ}\text{C}$. On the next day, 100 μl of tracer was added in similar manner. After 18–24 hours of incubation, the mixture was treated with 1 ml of the precipitating reagent , vortexed and left for incubation at $+4^{\circ}\text{C}$ for 20 min. After centrifugation of the tubes at 2 000 r.p.m (1 000 x g) for 20 min at $+4^{\circ}\text{C}$, the supernatant was separated from the pellet, and counted in the gamma counter for 5 min .

2.2.6.4. Glucagon-like peptide-1 radioimmunoassay.

For measurements of GLP-1 in plasma a single antibody technique with coated charcoal separation was utilized, as a modification of the method described by Orskov and Holst (1987).

Monoiodinated GLP-1 (^{125}I -GLP-1, specific activity $600\mu\text{Ci}/\mu\text{g}$), was prepared and diluted to give a final concentration of $0.26\text{ ng}/\mu\text{l}$. The antibody was raised in rabbits and directed against mid- to C-terminus of GLP-1 molecule (Affiniti Research Products Limited, UK).

GLP-1 in plasma was measured as follows: to $100\mu\text{l}$ of sample or standard, tracer (^{125}I -GLP-1, $100\mu\text{l}$), antibody ($100\mu\text{l}$) and glycine buffer (pH 8.8, $100\mu\text{l}$) were added. This mixture was vortexed and allowed to incubate at $+4^{\circ}\text{C}$ for 4 days. On the fifth day, separation of free and bound peptide molecules was accomplished by adsorption to dextran-coated charcoal (0.32% dextran, 0.25% gelatin). After vortexing, the tubes were centrifuged at 2 000 r.p.m ($1\ 000 \times g$) for 15 min at $+4^{\circ}\text{C}$. The supernatant was decanted into a counting tube and along with the pellet was counted in the gamma counter for 5 min.

2.2.6.5. Glucagon radioimmunoassay.

The analytical measurements of the levels of glucagon in plasma have been accomplished with the use of a double antibody technique and monoiodinated glucagon (^{125}I -glucagon), with specific activity less than $3\mu\text{Ci}$ per vial as a tracer. The guinea pig anti-glucagon pancreatic specific antiserum and goat anti-guinea pig IgG were used as a first and second antibody respectively. The carrier was identical with that used in insulin RIA (i.e. guinea pig normal carrier) and consequently its pre-experimental treatment as well as that of second antibody was much the same as in case of insulin radioimmunoassay. Preparation of the

label required addition of 25 ml of the glycine buffer (0.2M glycine, 0.03M EDTA, 1% BSA, 0.05% sodium azide; pH 8.8). The first antibody was also dissolved in the buffer. The tracer was then aliquoted to 2.8 ml volumes, whereas the remaining components were stored in 5 ml quanta at -20°C . All of the reagents were purchased from Linco Research Inc., St. Louis, MO.

The assay procedure involved addition of $100\mu\text{l}$ of the antibody and $100\mu\text{l}$ of glycine buffer (pH 8.8) to $100\mu\text{l}$ of sample or standard. After mixing and overnight incubation, $100\mu\text{l}$ of tracer was added. The content of the tubes was vortexed again and allowed to stand overnight at $+4^{\circ}\text{C}$. On the third day, $100\mu\text{l}$ of each carrier and second antibody were added. The mixture was vortexed and left to incubate at $+4^{\circ}\text{C}$ for 2 h. After that time, the samples were centrifuged at 2000 r.p.m ($1000 \times g$) for 15 min at $+4^{\circ}\text{C}$ and following separation, the pellet was counted in the gamma counter.

2.3. Calculations.

2.3.1. Assessment of tissue blood (plasma) flow.

At the level of the liver, the calculations were based on the measurements of the arterio-venous difference in plasma concentration of a dye, indocyanine green. For determinations of the hepatic extraction of indocyanine green (ICG), the following formula has been used:

$$\text{LPF} = (\text{ICG})_{\text{rate}} / \{[\text{ICG}]_{\text{A}} - [\text{ICG}]_{\text{Hv}}\}$$

(Caesar et al, 1961)

where:

LPF – Liver Plasma Flow,

$(ICG)_{rate}$ – Rate of ICG Infusion,

$[ICG]_A$ – Arterial Plasma Concentration of ICG,

$[ICG]_{HV}$ – Hepatic Venous Plasma Concentration of ICG.

At the level of peripheral tissue (hindlimb), direct perivascular flow measurements were obtained using the Transonic Flowmeter and were corrected to plasma flow, using measured values of hematocrit (see below).

2.3.2. Tissue fluxes of the metabolites and hormones.

For each organ considered in our study, the overall estimation of fluxes of glucose or hormones was based on two contributing factors :

- (i) A measurement of the Influx (I) of Substance X, which is the amount of the metabolite or hormone presented to the organ,
- (ii) A measurement of the Efflux (E) of Substance X, which is the amount of the metabolite or hormone leaving the organ.

The difference between the values of Influx into and Efflux from the organ in question gives the measure of the Net Uptake (NU) or Net Production (NP) of Substance X in the organ considered. This can be calculated as follows:

$$NU = I - E$$

$$NP = E - I$$

A rate of Fractional Extraction (%E) of Substance X by a particular organ can be determined according to the following formula:

$$\%E = 100 \times U / I,$$

when U is a positive quantity.

The equations presented below are used for calculations at the level of individual tissue beds.

2.3.2.1. Liver balances.

The amount of glucose or hormone (Substance X) presented to the liver is the sum of concentrations in the portal and hepatic veins; since portal blood flow is 75–80% of the total blood flow reaching the liver (Greenway & Stark, 1971). Therefore the following formula can be employed :

$$I = LPF (0.8 \times [X]_{PV} + 0.2 \times [X]_A)$$

$$E = LPF \times [X]_{HV}$$

where:

[X]_{PV} – Portal Vein Plasma Concentration of Substance X,

[X]_A – Arterial Plasma Concentration of Substance X,

[X]_{HV} – Hepatic Vein Plasma Concentration of Substance X

2.3.2.2. Splanchnic balances.

Splanchnic balance is calculated by subtracting hepatic venous from arterial concentration of Substance X in question, and multiplying this difference by the value of liver plasma

flow. The following equations are therefore used:

$$I = \text{LPF} \times [X]_A$$

$$E = \text{LPF} \times [X]_{HV}$$

2.3.2.3. Intestinal balances.

The rate of intestinal blood (plasma) flow is 0.8 of that in the liver. The net balance of fluxes of metabolites and hormones across the intestinal tissue is thus calculated as follows :

$$I = 0.8 \times \text{LPF} \times [X]_A$$

$$E = 0.8 \times \text{LPF} \times [X]_{PV}$$

2.3.2.4. Muscle balances.

Taking into account the hematocrit in converting blood flows to plasma flows in the hind-limb, the fluxes were calculated according to the formula :

$$I = \text{FBF} \times (1 - \text{Hct}) \times [X]_A$$

$$E = \text{FBF} \times (1 - \text{Hct}) \times [X]_{FV}$$

where:

FBF – Femoral Blood Flow,

[X]_{FV} – Femoral Vein Plasma Concentration of Substance X.

2.3.3. Assessment of insulin secretion.

A large and variable first-pass extraction of insulin occurs in the liver (Blackard & Nelson, 1970; Field, 1973 ; Eaton et al., 1980; Polonsky et al., 1983) following its secretion into the portal vein. Insulin levels cannot therefore be used in calculations of secretion. Contrary to the case of insulin, the hepatic extraction of the connecting peptide (C-peptide) appears to be negligible (Blackard & Nelson, 1970; Stoll et al., 1970; Eaton et al., 1980; Polonsky et al., 1983). Therefore the calculation of Insulin Secretion (IS) is based on the C-peptide appearance across the splanchnic bed. This is expressed by the equation:

$$IS = LPF \times ([C-P]_{HV} - [C-P]_A)$$

where:

$[C-P]_{HV}$ – Hepatic Venous Plasma Concentration of C-Peptide,

$[C-P]_A$ – Arterial Plasma Concentration of C-Peptide.

2.3.4. Data analysis and statistics.

Results were expressed as a mean and standard error of the mean.

2.3.4.1. Comparison of arterial metabolite and hormone concentrations.

Measured concentrations of hormones and metabolites (glucose, lactate, insulin, glucagon, C-peptide and GLP-1) in time were interpolated at specific time points by fitting the data to smoothing curves (Radziuk et al., 1978 b). The rationale for employing this technique was that the samples were not always obtained exactly at the scheduled time. The smoothed

data at each time point was then measured for the two groups considered: GLP-1 infusion (n=5) and control (saline infusion, n=5). The two groups were compared using a general linear models procedure (SAS Institute) using time as a repeated measure. Differences between the curves were compared for the two groups (GLP-1 and control) at the three levels:

- (1) TREATMENT – values (means \pm SEM) were pooled together for GLP-1-treated groups and for the control groups. This comparison indicated whether any differences existed between the two groups.
- (2) TIME – data points (means \pm SEM) for both groups (GLP-1 and controls) were pooled at each time point and the possibility of a change in time in the data set was examined.
- (3) TIME \times TREATMENT – this interaction term determined whether GLP-1 and control groups changed in time, but differently.

A posteriori (post-hoc) paired comparisons between data for each group (TREATMENT) at individual time points were also made. The level of statistical significance was set at $p < 0.05$ (Cody & Smith, 1991; Kitchens, 1987; Dawson-Saunders & Trapp, 1994).

2.3.4.2. Comparison of tissue balances.

Net tissue balance was calculated as discussed above for glucose, lactate, C-peptide and insulin for appropriate tissues. This is presented either as extraction (%) or uptake (mass units/kg/min). Evaluations of tissue balance were made based on three sets of samples taken at the end of each experimental period (basal, glucose infusion alone, GLP-1 infusion at 4 ng/kg/min or at 8 ng/kg/min, see above). Saline was infused at comparable rates in the

control studies. Each set of samples was used in evaluation of the relevant tissue balances. The three sets were then averaged to obtain an estimate of the tissue flux considered during each period of study. Again, the balances for each group (GLP-1 infusion or control) were averaged for each period. Analysis was done using the general linear model with time as repeated measure (SAS Institute) (Cody & Smith, 1991). Comparisons were made between groups (TREATMENT) for each data set as well as within each period. The effect of (TIME) and the (TREATMENT x TIME) interaction was also evaluated.

3. RESULTS

Results obtained in this study were based on experimental data from 10 successful experiments, five of which involved administration of GLP-1, and five – saline. Since the effects of GLP-1 are glucose-dependent, as indicated earlier, these studies were performed at elevated glucose levels. For this purpose, a hyperglycemic clamp was performed. Glucose concentrations were sampled frequently and the rate of glucose infusion (20% Dextrose) was adjusted accordingly to maintain glucose concentration near 150 mg/dl.

3.1. Metabolite concentrations.

Figure 1 shows the plasma glucose concentrations obtained during these studies. There was no significant difference between the glucose concentrations obtained during experiments where GLP-1 was infused or during control studies where only an equivalent amount of saline was administered. Fasting glucose concentrations in these animals were somewhat different at the first two data points. As will be seen, however, this difference did not appear to affect subsequent results (see glucose infusion rates).

Figure 2 shows the plasma lactate concentrations for the two sets of studies. Lactate concentrations are seen to be somewhat labile with fluctuations in concentration which do not appear to be related to the study protocol. These fluctuations may result from variety of factors (e.g., animal movement or small degree of stress). It can be seen from the statistical analysis, that the lactate concentrations for the two treatments (GLP-1 infusion or saline infusion in controls) were not significantly different.

Figure 1.

The plasma glucose concentrations are shown under basal (fasting) conditions: ($t < 0$), during the infusion of glucose alone (hyperglycemic clamp) ($0 < t < 90$ min) and during the additional infusion of GLP-1 at 4 ng/kg/min ($90 < t < 180$ min) and 8 ng/kg/min ($180 < t < 270$ min). The analysis of variance table is given below. Glucose concentrations during the clamp were statistically identical.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	2.35	0.17
TIME	94.4	0.0001
TIME x TREATMENT	0.97	0.51

Plasma glucose

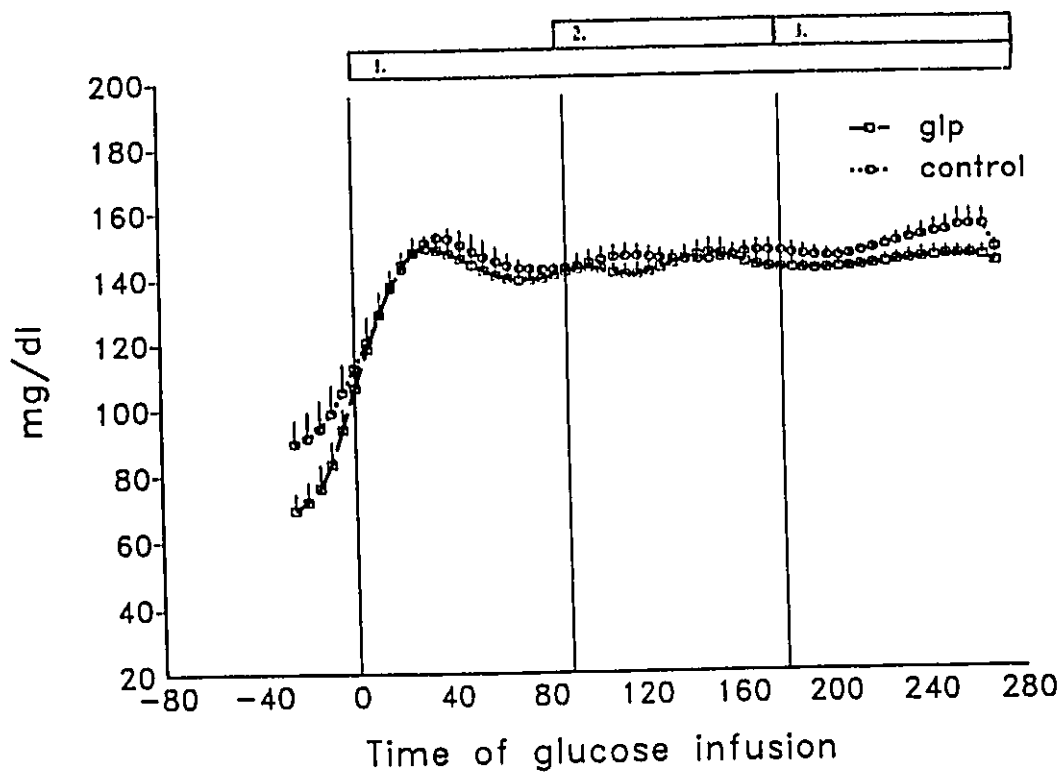


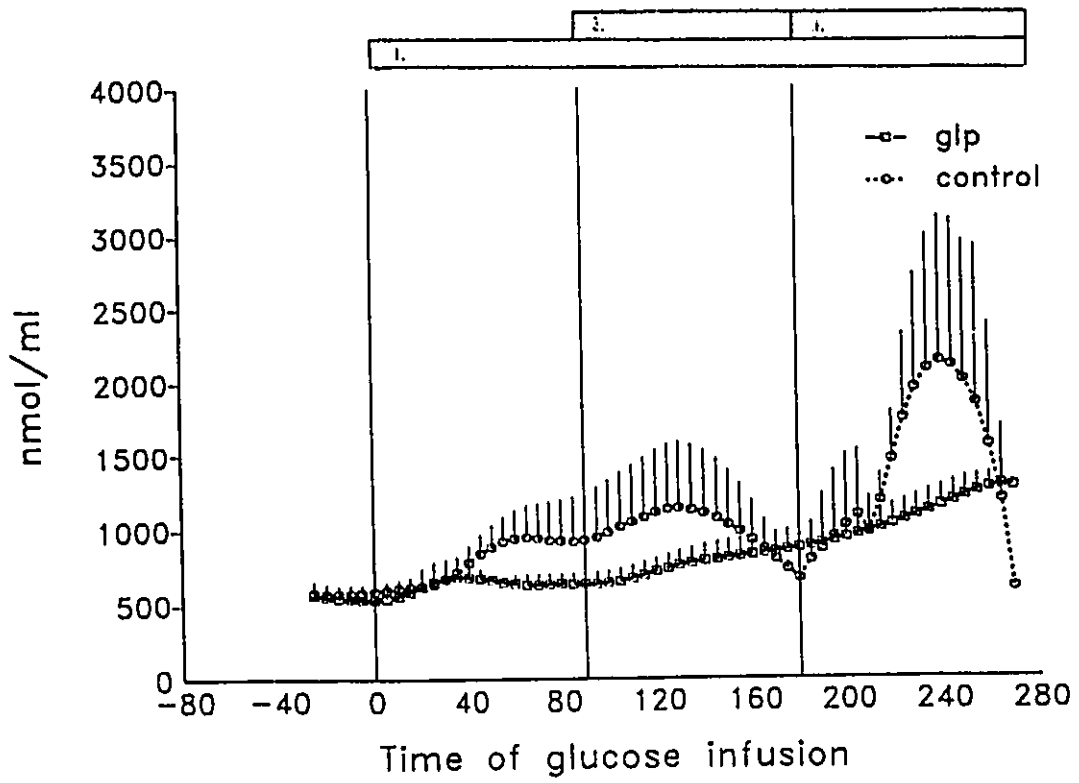
Figure 2.

The changes of plasma levels of lactate (nmol/ml) are presented for both experimental protocols. The analysis of variance given below indicates that there are statistically significant changes occurring in time ($p < 0.0001$), likely related to the duration of the hyperglycemic clamp. Lactate concentrations seen in the case of the control group appear to be quite unstable, but this does not appear to be related to the study protocol and statistical analysis does not reveal any significance in this observation.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	1.7	0.24
TIME	7.2	0.0001
TIME x TREATMENT	1.3	0.19

Plasma lactate



3.2. Hormone concentrations.

Figure 3 demonstrates the GLP-1 concentrations under fasting conditions, during glucose infusion alone and during administration of GLP-1 at 4 ng/kg/min and 8 ng/kg/min. Fasting GLP-1 concentrations averaged at 62.4 ± 16.3 pg/ml. During the glucose infusion, concentrations increased to 113 ± 29 pg/ml, averaging near 90 pg/ml throughout most of the study. During infusion of GLP-1 at 4 ng/kg/min, concentrations increased to 312 ± 21 pg/ml, followed by a second elevation to 565 ± 58 pg/ml during the 8 ng/kg/ml infusion. It should be noted that the increment in GLP-1 concentrations is approximately 240 pg/ml during the first infusion, and 490 pg/ml during the second infusion of GLP-1. This indicates highly linear kinetic behaviour on the part of GLP-1 at these concentrations. There is no evidence of saturation in elimination mechanisms.

Figure 4 demonstrates the changes in plasma C-peptide in response to GLP-1 infusion compared to control. The two C-peptide curves were not different (at a level of significance $p=0.05$) for the two treatment groups. An effect of time was however readily discernible ($p=0.0001$). Moreover, an interaction of treatment with time is also highly evident in this case, indicating that there is a divergence of the two sets of results with time. Pairwise comparison at successive data points indicates that this divergence becomes significant at time $t=160$ min. These data therefore indicate that there is an effect of GLP-1 infusion on C-peptide concentrations, implying that GLP-1 does indeed have an effect on insulin secretion.

Figure 3.

The plasma concentrations of GLP-1 at fasting conditions, followed by periods (90 min each) of glucose, and glucose supplemented with GLP-1 (4 ng/kg/ min and 8 ng/kg/min) infusions. Statistical differences were observed in time as well as between the GLP-1 and control groups (TREATMENT). Divergence between the two curves became significant at $t > 105$ min.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	22.7	0.002
TIME	41.5	0.0001
TIME x TREATMENT	34.3	0.0001

Plasma GLP-1

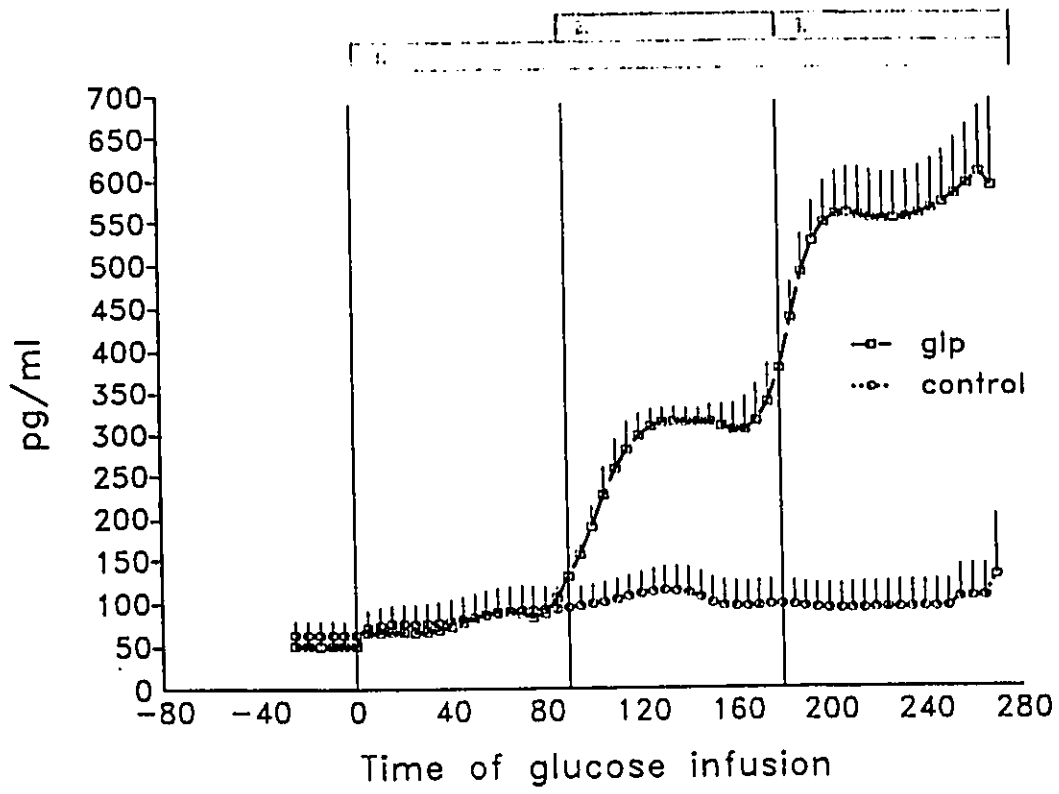


Figure 4.

The alterations in plasma levels of C-peptide secreted during the four stages of experiment (basal, glucose, two-step GLP-1 infusion). Statistical differences are readily evident for the effect of time ($p < 0.0001$), as well as for the interaction of the treatment with time ($p < 0.0001$). Divergence of the C-peptide concentration curves became significant at $t = 160$ min. The statistical analysis is presented below.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	5.2	0.06
TIME	15.4	0.0001
TIME x TREATMENT	8.6	0.0001

Plasma C-peptide

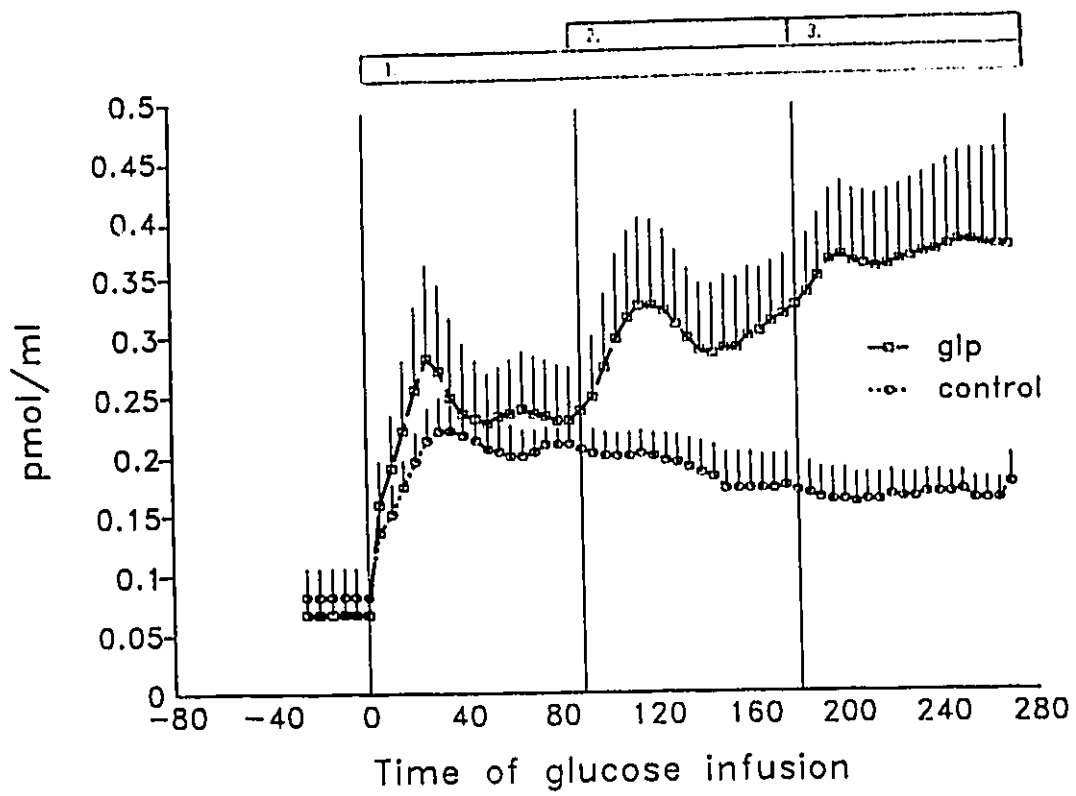


Figure 5 demonstrates the insulin concentrations during studies with GLP-1 infusion and the analogous controls, where saline was administered instead. In the control study, insulin concentration was $9 \pm 1 \mu\text{U}$ during the basal (fasting) period. This increased to peak of $19.2 \pm 2 \mu\text{U/ml}$ soon after initiation of the glucose infusion. There was a subsequent fall to $14 \pm 1 \mu\text{U/ml}$, and after that insulin concentrations remained between 14 and $17 \mu\text{U/ml}$ for the duration of the glucose infusion. With the superimposition of GLP-1 infusion, instead of decreasing, insulin concentrations continued to rise to a maximum level of $28 \pm 2 \mu\text{U/ml}$. The effect of GLP-1 infusion (TREATMENT) and time progress (TIME) are highly significant. A divergence between the two curves also appears in time. Pairwise comparisons further indicate that insulin concentrations become significantly different for the time $t > 65 \text{ min}$. That this appears to occur prior to the initiation of GLP-1 infusion is likely due to the effects of smoothing and interpolation of data points.

Figure 6 demonstrates plasma glucagon concentrations under basal conditions, during glucose infusion alone and during glucose infusion supplemented with GLP-1 infusion. There appears to be a slight suppression of glucagon concentrations under both sets of conditions, however this is not statistically significant. There are also no differences evident between the two sets of data. From this data, it is not likely that differences in the suppression of glucagon play a part in inducing the differences in metabolic effects which are seen during GLP-1 infusion.

Figure 5.

Insulin is co-secreted with C-peptide on an equimolar basis. This is reflected in the graphs which illustrate the plasma concentrations of insulin during the studies. Similarly to the C-peptide statistics, significant changes are noted to occur in time. Moreover the effect of GLP-1 infusion (TREATMENT) is also significant. The divergence of the curves for the control and GLP-1 protocols occurs at t=65 min. These statements are supported by results of statistical analysis provided below.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	7.8	0.03
TIME	14.0	0.0001
TIME x TREATMENT	3.7	0.0001

Plasma insulin

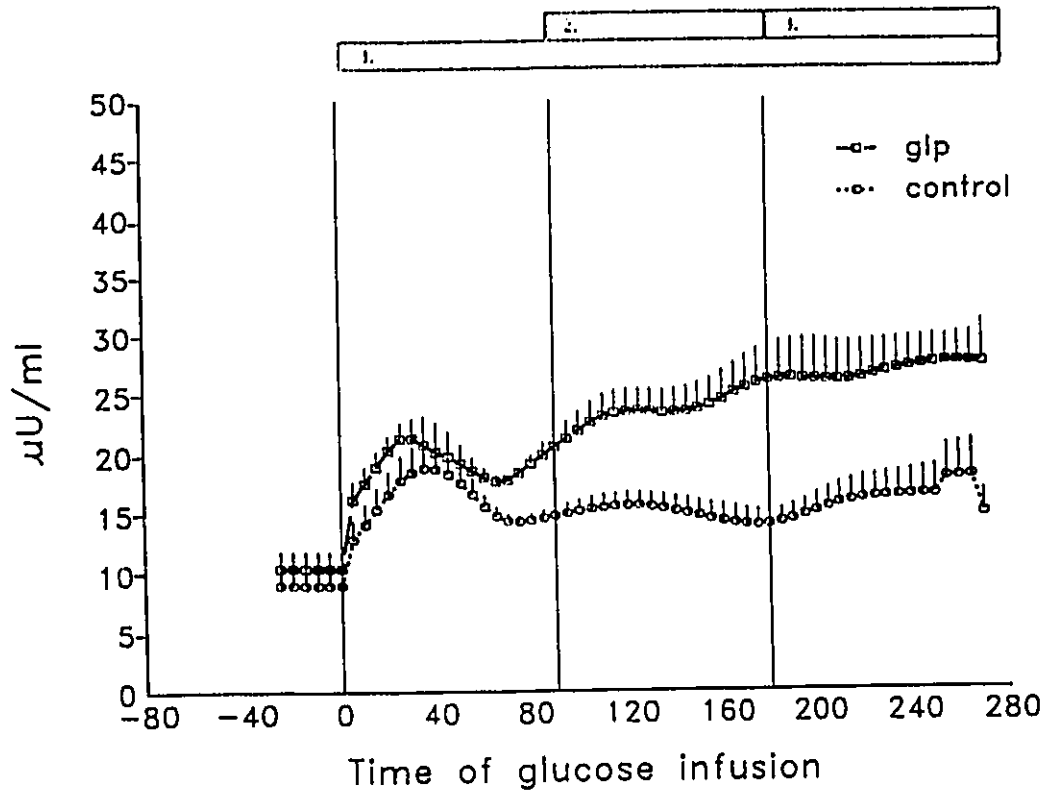


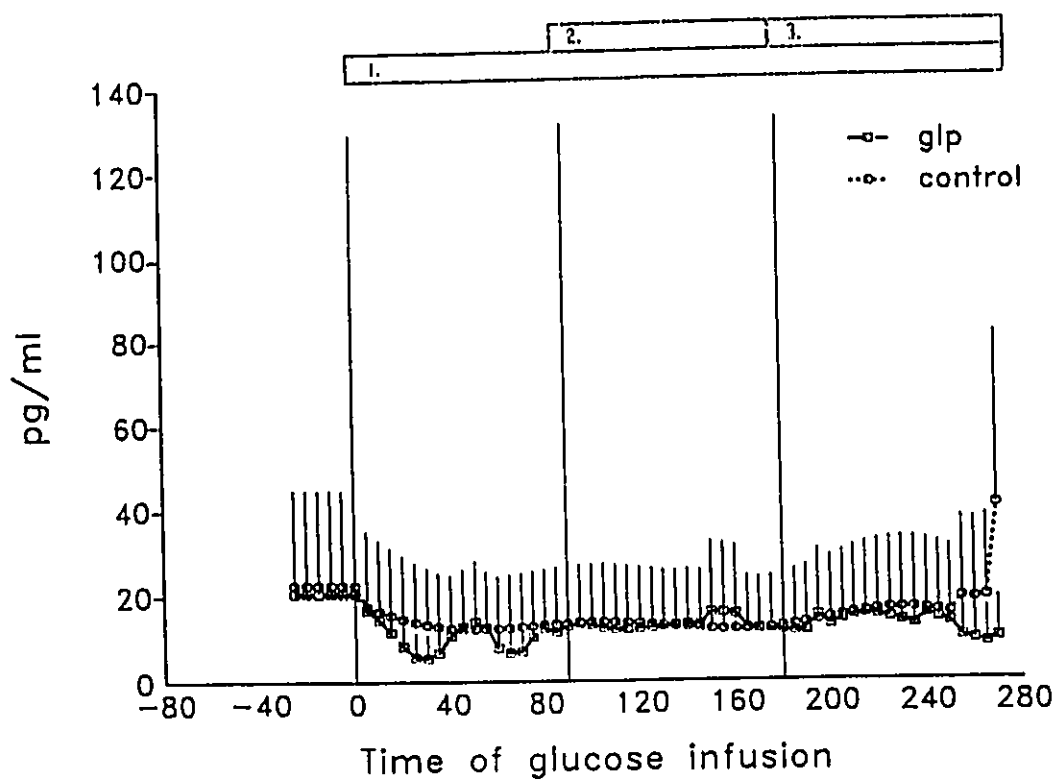
Figure 6.

The changes in glucagon concentrations during the basal period, and throughout glucose infusion alone or with GLP-1 administration superimposed on glucose infusion. Because of the variations in glucagon concentrations, the suppression of glucagon during the study does not appear to be significant. Statistical analysis indicates that there was no overall difference between the two sets of the study groups (at the level of $p < 0.05$).

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.1	0.78
TIME	1.4	0.16
TIME x TREATMENT	0.9	0.68

Plasma Glucagon



3.3. Glucose requirements.

Figure 7 demonstrates the rate at which glucose needed to be infused during both sets of studies in order to maintain the clamp level of 150 mg/dl. Statistical analysis indicates that there was no overall difference between the glucose requirements with or without GLP-1 infusion. There is however, according to the performed statistical analysis, a highly significant effect of time, as well as an interaction of time and treatment. The latter indicates that the glucose requirements in the two groups divert with time differently for the two treatments. This is compatible with the GLP-1 infusion which occurs only after the time $t = 90$ min. Pairwise comparison at the different time points indicates that significance is attained for $t > 225$ min. These data therefore clearly indicate an overall difference of amounts of glucose required to maintain the hyperglycemic clamp when GLP-1 was infused, only during the rate of 8 ng/kg/min.

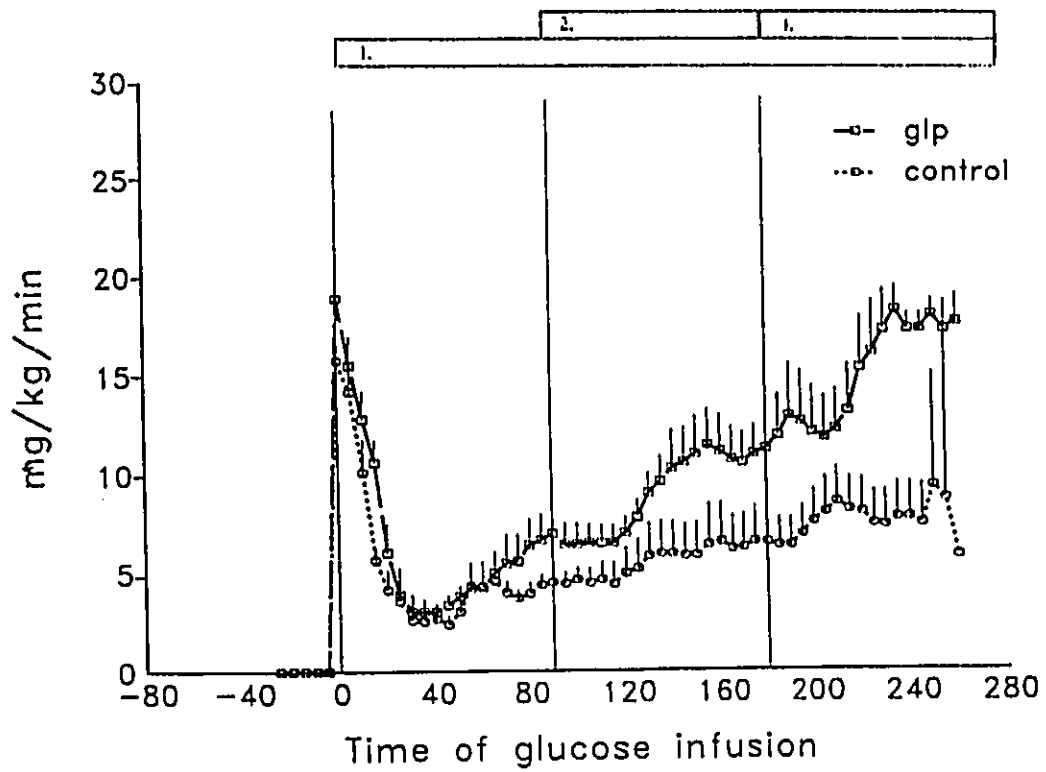
Figure 7.

Rate of glucose infusion as a reflection of glucose requirements needed to maintain glucose levels at 150 mg/dl. There is an increase in glucose requirements in time. The difference between the two curves attains statistical significance and divergence occurs in time as GLP-1 infusion rate is increased. Pairwise comparison of successive data points indicates that this divergence occurs for $t > 225$ min.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	2.5	1.0
TIME	14.4	0.0001
TIME x TREATMENT	1.9	0.01

Rate of glucose infusion



3.4. Tissue balances of metabolites and hormones.

Figure 7 suggested that increased glucose requirements are necessary during the GLP-1 infusion. However, from an overall systemic point of view, these differences in requirements were not as strong as the differences in insulin and C-peptide concentrations might suggest. More sensitive indicators of the changes in metabolic effects induced by GLP-1 infusion might therefore be obtained by examining these effects at the target tissue levels. In response to acute changes in insulin, glucose is removed primarily by peripheral tissues (Radziuk & Inculet, 1983), characterized by the muscle of the hindlimb in this case. The liver may also respond to changing insulin levels, by both reducing the rate of glucose production as well as increasing the rate at which it takes glucose up from the circulation (Steele et al., 1965; Felig and Wahren, 1971). The gut also utilizes insulin and may under some circumstances be responsive to insulin or other hormones. Metabolite, specifically glucose, balance was therefore examined across these three tissue beds. Because the analysis involves small differences between large numbers, the data are necessarily noisier. It is expected that only large and therefore important effects should be discernible with this analysis.

3.4.1. Glucose balances.

Figures 8 and 9 demonstrate glucose balances across individual tissue beds as determined in this study.

Figure 8 (top panel) demonstrates the splanchnic balance of glucose. Glucose uptake is in the positive direction. It can be seen that under fasting conditions the splanchnic bed is a

net producer of glucose. It is clearly the difference between liver production (lower panel) and intestinal uptake. During the period of initial glucose infusion, the results are somewhat ambiguous. The reason for this is that large and rapid changes in glucose metabolism by the tissues are occurring during this period. Under these conditions arterio – venous differences are not as accurate as under conditions which were closer to steady state. There is, however, a clear shift from net production (GLP-1: 1.3 ± 0.1 versus control 1.6 ± 0.4 mg/kg/min) to net uptake as glucose infusion progresses (4.2 ± 2.1 versus 3.3 ± 1.5 mg/kg/min for GLP-1 versus control during the second GLP-1 infusion period).

The liver (lower panel) reflects the overall changes seen in splanchnic bed. During fasting, glucose production is higher (2.3 ± 0.4 and 2.3 ± 0.5 mg/kg/min). While GLP-1 infusion progresses, this converts to an uptake of 1.1 ± 0.3 and 1.4 ± 0.5 mg/kg/min for GLP-1 and control studies respectively. There is a significant effect of time (and therefore of continued glucose infusion) in the liver balance only. Pairwise comparison also indicates that in the period when glucose was infused alone, there is a significant difference between the two studies. This difference can be discounted however since during this period differences are less valid as the rapid changes in glucose fluxes lead to a non-steady state situation

Figure 8 – top panel.

This panel indicates mean splanchnic balance of glucose calculated for the basal period, the period of initial glucose infusion, as well as two levels of GLP-1 infusion: 4 ng and 8 ng/kg/min. Data for GLP-1 infusion are compared to control data where an equivalent amount of saline was infused. Splanchnic balance was calculated by subtracting hepatic venous glucose from arterial glucose and multiplying this difference by hepatic blood flow. The analysis of variance table given below indicates that there are no significant differences either in time or between the two treatments for the study groups.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.03	0.87
TIME	6.0	0.09
TIME x TREATMENT	0.69	0.62

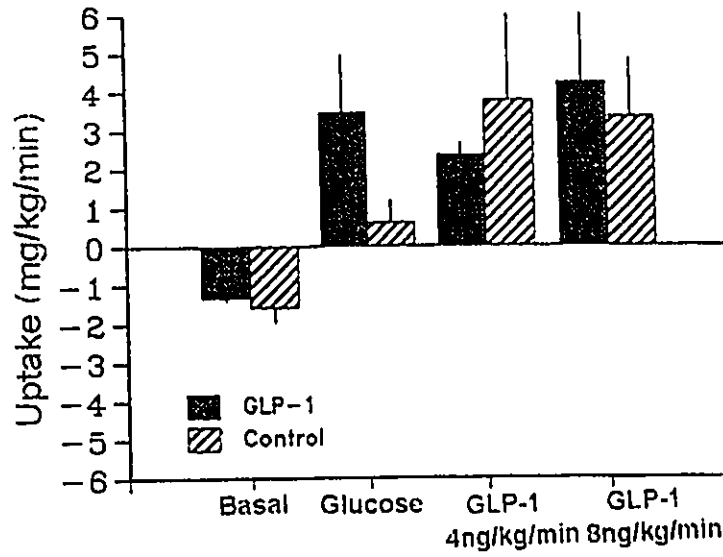
Figure 8 – lower panel.

Liver balances of glucose are shown calculated as indicated above except that inflow of glucose to the liver is determined from both its portal concentration (80%) and arterial concentration (20%). The outflow is again determined by hepatic venous glucose concentrations. The difference between inflow and outflow is multiplied by total hepatic venous glucose concentrations. The analysis of variance (below) indicates that there is a progression in time in the liver balance, which is clearly from the net production to net uptake, but this is not different for the two treatments used in these studies (GLP-1 infusion and saline infusion in the control case).

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.47	0.52
TIME	14.9	0.01
TIME x TREATMENT	3.20	0.15

GLUCOSE Splanchnic balance



GLUCOSE Liver balance

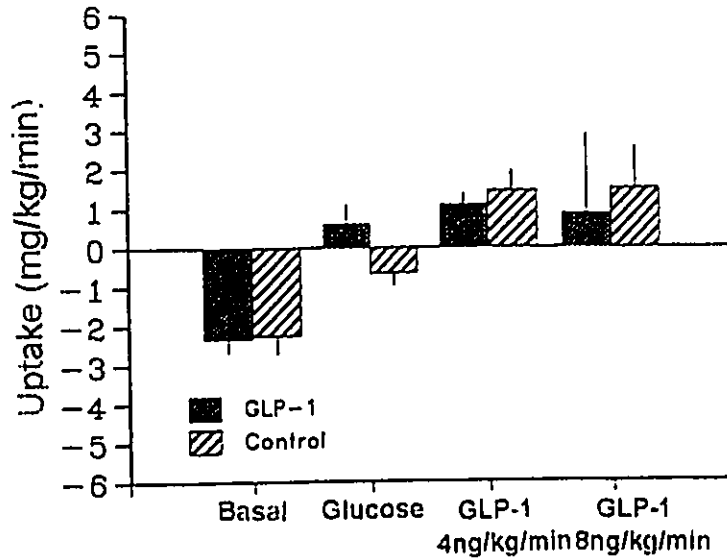


Figure 9 (top panel) demonstrates intestinal balances of glucose during the four periods of study. Again, the only significant effect is due to continuing glucose infusion in time. There are no differences between the two treatments (GLP-1 and control).

The hindlimb (lower panel) represents the peripheral tissues, primarily muscle in these studies. From this figure it is evident that GLP-1 has an important effect on uptake at the level of this tissue. There is a significant effect of time on femoral glucose uptake. There is also a highly significant effect of treatment – that is, GLP-1 infusion versus control (saline administration). There is, however, no interaction between treatment and time, suggesting that there is a progressive increase in hindlimb glucose uptake in both cases (GLP-1 and control protocols). However, the treatment difference suggests that although these changes are occurring in a manner which cannot be distinguished from parallel, they are higher for the studies where GLP-1 infusion was administered. That this is indeed the case is also seen in the fact that pairwise comparisons indicate significant differences only during the last two periods of study (GLP-1 infusion at 4 ng and 8 ng/kg/min). These were 0.11 ± 0.06 and 0.18 ± 0.14 mg/kg/min for the control studies and 0.41 ± 0.10 and 0.50 ± 0.06 mg/kg/min for the GLP-1 infusions.

Figure 9 – top panel.

Glucose balances across the intestinal tissue are depicted for the four stages of experiment: basal, glucose infusion alone and glucose infusion with superimposed GLP-1 infusions (two rates). This was calculated from differences in arterial and portal plasma concentrations of the metabolite and further multiplication by values of intestinal plasma flow. The analysis presented below indicates that the only significant effect of glucose fluxes across the gut is exerted with progression of time. There is a statistically significant increase in intestinal uptake in time but no effect which can be attributed to the addition of a GLP-1 infusion.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.94	0.37
TIME	6.5	0.05
TIME x TREATMENT	1.3	0.38

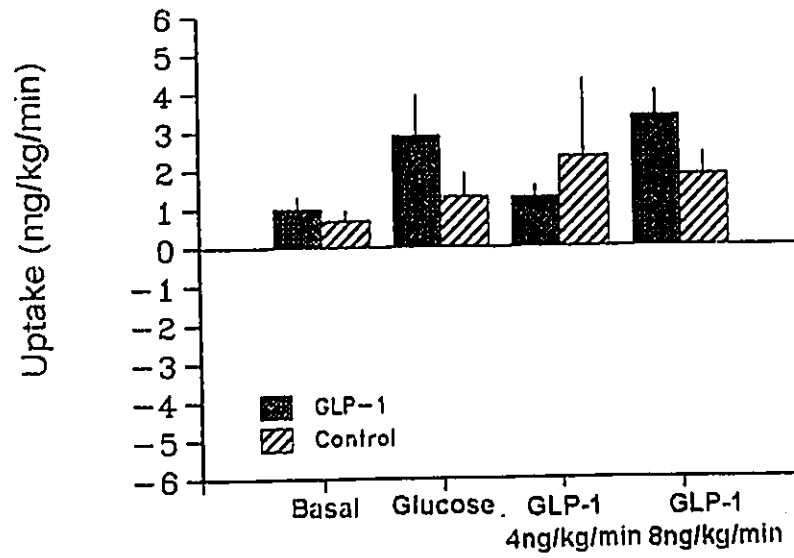
Figure 9 – lower panel.

Glucose balances, expressed as uptake of this metabolite by the hindlimb are indicated for consecutive stages of the experiments. The hindlimb (femoral) fluxes of glucose were determined from arterio-venous differences in metabolite concentrations, which were subsequently multiplied by values of femoral plasma flow (derived from readings of femoral blood flow corrected using the hematocrit). Significant differences were observed for the two protocols: GLP-1 administration and control. Pairwise comparisons between corresponding values indicate most differences during the last two (GLP-1 infusions) periods. These results therefore indicate that GLP-1 indeed has important effects on muscle uptake.

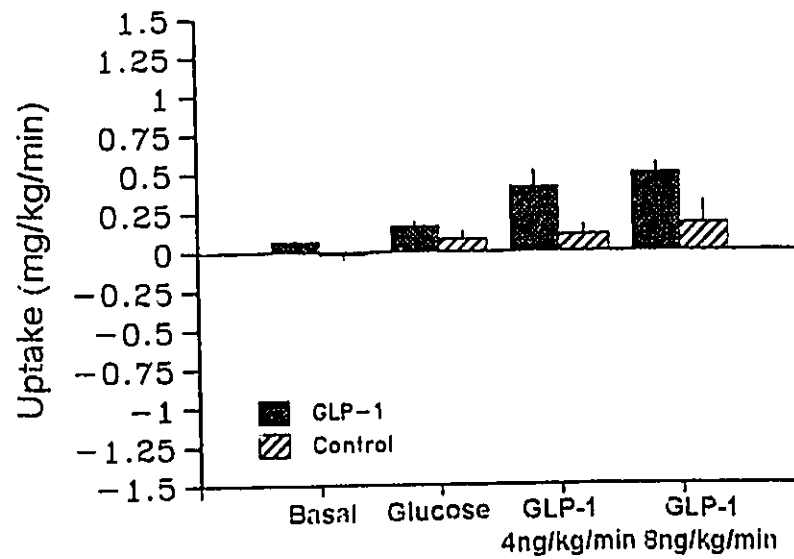
ANALYSIS OF VARIANCE

	F	P
TREATMENT	8.8	0.02
TIME	5.2	0.05
TIME x TREATMENT	2.5	0.17

GLUCOSE Intestinal balance



GLUCOSE Femoral balance



3.4.2. Lactate balances.

Figure 10 and 11 demonstrate lactate balances in these studies.

Figure 10 gives both the splanchnic and liver balance of lactate expressed as fractional extractions. Lactate extraction by the liver (and therefore by the splanchnic bed overall) has traditionally been found to be near 50% in the pig (Radziuk, 1987). These previous observations are supported in these data. For example, fasting extraction is $57\pm 5.6\%$ and $52\pm 15\%$ in the control and GLP-1 infusion experiments respectively. This rate of extraction appears to drift down during the glucose infusion, but the effect is not significant in either the liver or the splanchnic bed overall. There are also no statistically significant effects determined on hepatic or splanchnic lactate balance due to the GLP-1 infusion.

Figure 11 indicates that both in the intestinal bed and in the hindlimb, there is an overall lactate production, at least under fasting conditions and during most of the glucose infusion. There are no significant changes in time of this lactate balance nor are there effects of GLP-1 infusion discernible.

Lactate uptake by the splanchnic bed and in particular the liver is primarily for the gluconeogenic production of glucose or glycogen. Clearly, neither gluconeogenesis nor glycogenesis from lactate is affected in any major way by the infusion of GLP-1. If the production of lactate is considered as an indicator of glycolysis in the gut or hindlimb, again, there are no discernible changes in the process due to the GLP-1 infusion.

Figure 10 – top panel.

Splanchnic balance of lactate expressed as fractional extraction is presented at four consecutive stages of experiment. Extraction was calculated from the ratio of uptake divided by influx of metabolite. The tendency of lactate extraction across the splanchnic bed to decrease is not statistically significant. There was also no difference between the two study groups. The results of analysis are provided below.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.65	0.4
TIME	4.09	0.1
TIME x TREATMENT	5.42	0.07

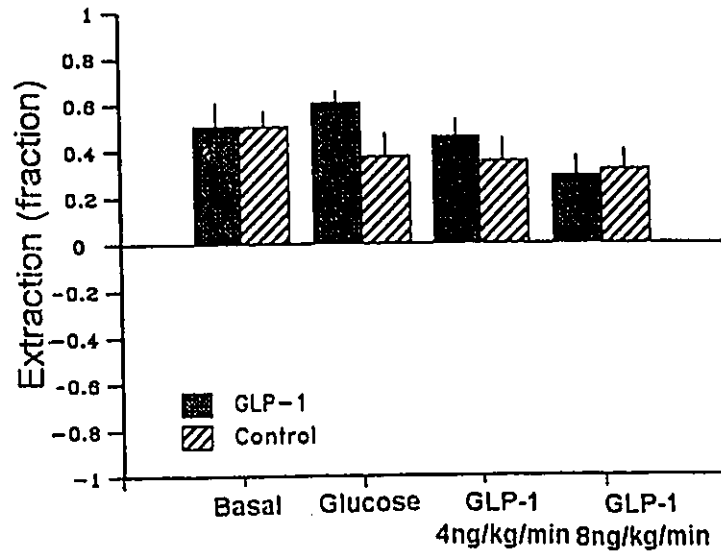
Figure 10 – lower panel.

Lactate balances across the liver are depicted under basal conditions and during infusion periods, as determined by protocol. The liver extraction of metabolite was determined from the values of its uptake divided by influx of lactate into this organ. The tendency for lactate extraction to decrease (analogous to the situation in the splanchnic bed) is not statistically significant. Nor are there any differences which can be attributed to the GLP-1 infusion.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.38	0.56
TIME	2.89	0.17
TIME x TREATMENT	2.81	0.17

LACTATE
Splanchnic balance



LACTATE
Liver balance

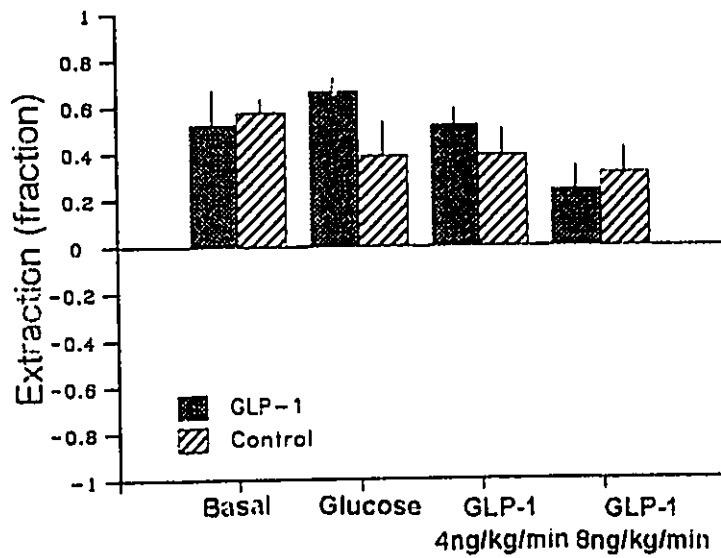


Figure 11 – top panel.

Lactate balance across the intestinal tissue presented as uptake at each stage of the experiment. Negative values of metabolite uptake indicate its net production. The analysis of variance (below) shows no significant differences of lactate balance during the study or between the two groups.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	0.02	0.88
TIME	3.33	0.14
TIME x TREATMENT	1.61	0.32

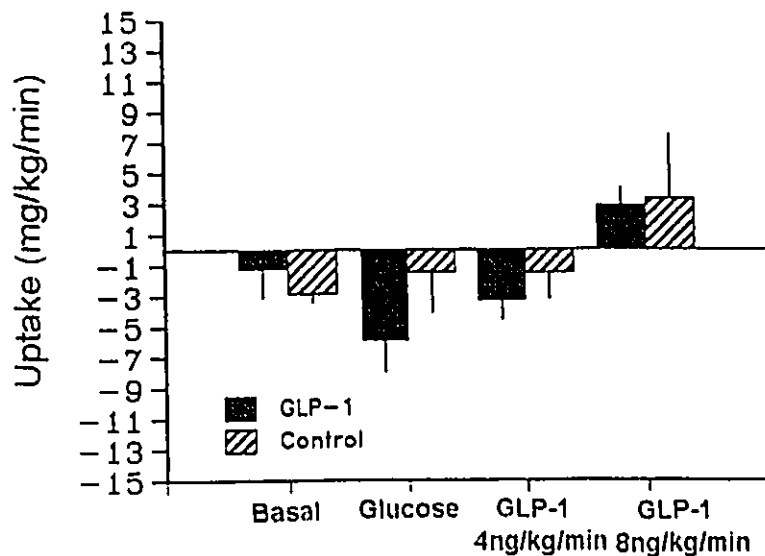
Figure 11 – lower panel.

Lactate balance expressed as an uptake of this metabolite across the peripheral tissue (hindlimb) determined for the basal period as well as for the subsequent periods of glucose and GLP-1 infusions. Again, negative values of lactate uptake throughout most of the study indicate that net production is taking place. Statistical analysis demonstrates lack of significant differences between the groups studied as well as no influence of time on lactate fluxes across the hindlimb.

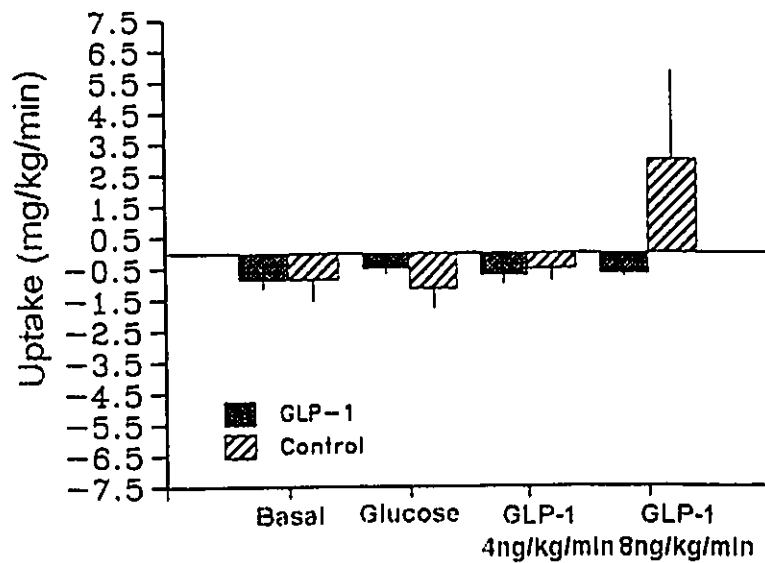
ANALYSIS OF VARIANCE

	F	P
TREATMENT	4.5	0.08
TIME	0.32	0.80
TIME x TREATMENT	4.7	0.09

LACTATE Intestinal balance



LACTATE Femoral balance



3.4.3. C-peptide balances.

Since first-pass C-peptide uptake by the liver is generally considered to be negligible as previously indicated, the splanchnic balance of this peptide can be used as a direct indicator of insulin secretion. Problems are associated with this type of measurement since insulin secretion tends to be pulsatile (Stagner & Samols, 1988), leading to noise in the data. This is attenuated as much as possible by averaging three samples during each period.

Figure 12 demonstrates a significant increase in C-peptide release from the splanchnic bed in time (i.e. during progressive glucose infusion). There is not, however, an apparent significant difference in splanchnic C-peptide release between the two treatments. However, comparison of C-peptide release during the final (8 ng/kg/min) period of GLP-1 infusion indicates that this is significantly higher for the GLP-1 infusion as compared to the control ($p = 0.015$). Indeed, this is 4.5 ± 0.7 pmol/kg/min for the GLP-1 infusion and 1.65 ± 0.62 pmol/kg/min for the control situation. These data are completely compatible with the concentration differences seen in Figure 4, where it was indicated that C-peptide levels during the GLP-1 infusion became significantly different only near the end of the first step of the infusion of GLP-1. Nevertheless, within the limitations of these data, these results taken together indicate an unequivocal increase in insulin secretion in response to GLP-1 infusion in this animal model.

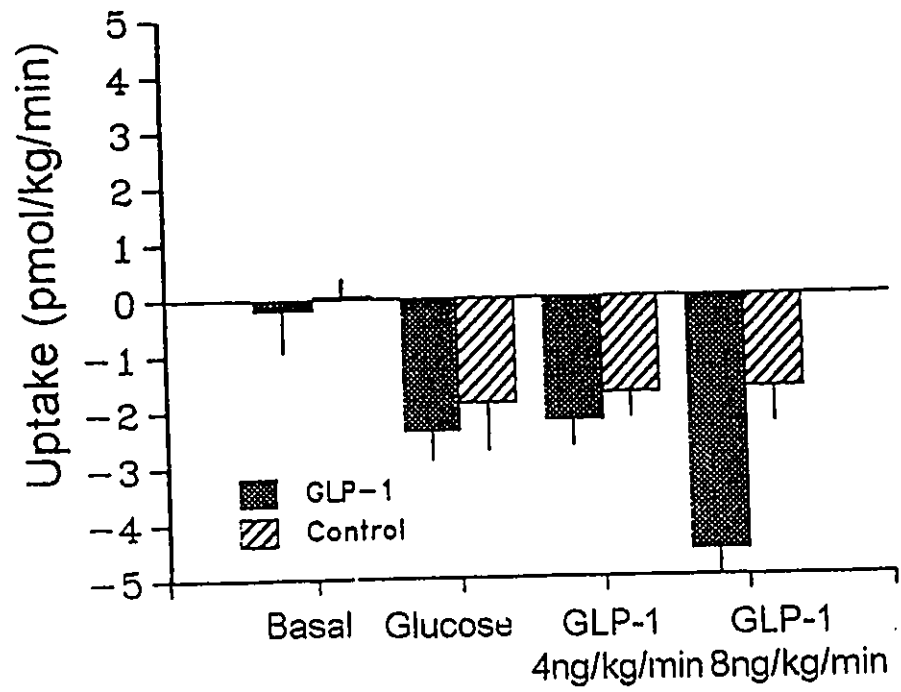
Figure 12.

There is an overall increase in C-peptide secretion (negative uptake) during the course of the study. Although no overall effect between the two groups is discernible, the change in time can be attributed to an increase in C-peptide (insulin) secretion due to GLP-1 infusion, which is seen in the last period (GLP-1: 8 ng/kg/min). This is compatible with data from table 4.

ANALYSIS OF VARIANCE

	F	P
TREATMENT	3.6	0.12
TIME	19.0	0.02
TIME x TREATMENT	3.3	0.18
GLP-1 (8 ng/kg/min)	13.2	0.015

C-PEPTIDE Splanchnic balance



3.4.4. Insulin balances.

It is frequently suggested that insulin action may be related to its net uptake by its target tissues.

Figure 13 therefore was used to assess the uptake of insulin by the hindlimb, where it is considered to exert its major effects. From this figure, however, it is clear that there are no effects of GLP-1 on the uptake of insulin by the tissue of the hindlimb. Moreover, the hindlimb uptake of insulin under the circumstances of the hyperglycemic clamp are seen to be small (approximately 5% when measurable) and likely do not contribute to overall insulin action under these circumstances.

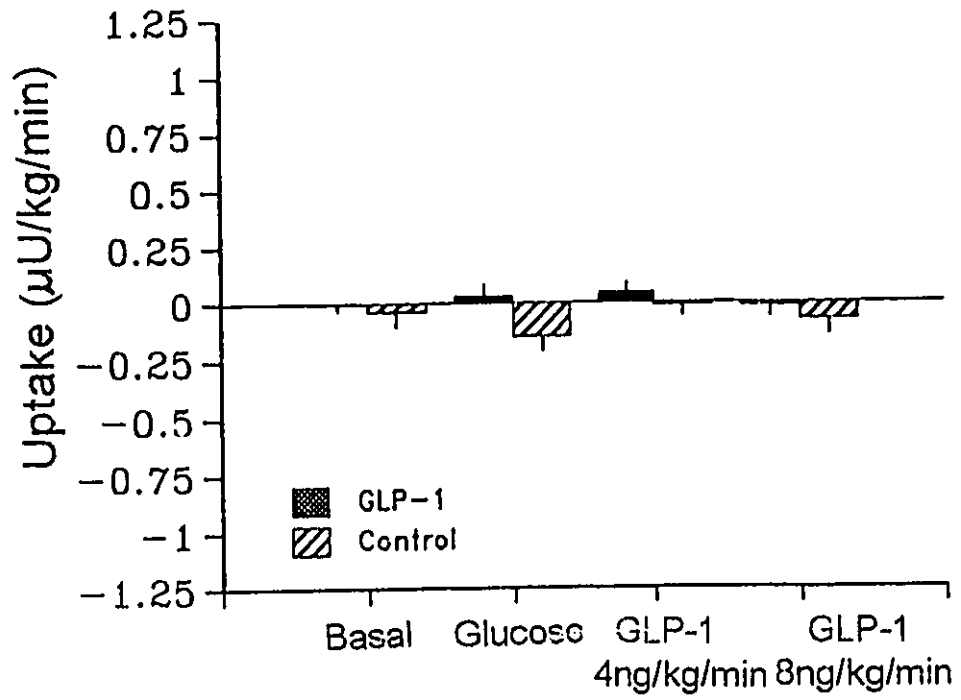
Figure 13.

The uptake of insulin by the peripheral tissue (represented by hindlimb) during the four stages of the experimental protocol. It is apparent that insulin fluxes in muscle are small, thus precluding the likelihood that GLP-1 might exert a major effect on insulin uptake by this tissue. This is confirmed statistically. No differences are seen either for treatment with GLP-1 infusion (TREATMENT) or with time progress (TIME).

ANALYSIS OF VARIANCE

	F	P
TREATMENT	1.09	0.3
TIME	0.47	0.7
TIME x TREATMENT	1.49	0.3

INSULIN Femoral balance



4. DISCUSSION

It has long been known that tolerance to oral glucose is improved as compared to that administered intravenously (Elrick et al., 1964; McIntyre et al., 1965). This improvement has been attributed, at different times, to a variety of factors. The hypothesis recognizing the central role of the liver in maintaining blood glucose homeostasis has been introduced by Bernard in 1878 (Bernard, 1878). The mechanism by which the liver might be able to differentiate between endogenous (oral) and exogenous (intravenous) glucose load has been extensively investigated. Hyperglycemia (Soskin & Levine, 1952; DeFronzo et al., 1978; Liljenquist et al., 1979; Sacca et al., 1982), elevated insulin concentrations (Felig & Wahren, 1971; DeFronzo et al., 1978; Cherrington et al., 1982; Sacca et al., 1982), insulin–glucagon interaction (Bomboy et al., 1977), gut–derived factors (Elrick et al., 1964; McIntyre et al., 1965; DeFronzo et al., 1978) and even neural stimuli (Novin et al., 1973; Adkins–Marshall et al., 1992; Moore et al., 1993) were among those factors most frequently suggested.

The physiological route of glucose entry by way of the portal vein was long considered to contribute significantly to this improved tolerance (Scow & Cornfield, 1954). Glucose which enters the portal vein following absorption immediately encounters the liver. During periods of glucose excess, the liver takes up glucose. It was hypothesized that, since after oral glucose, portal glucose levels were much higher than after intravenous glucose, this would promote the hepatic uptake of glucose. Indeed, Felig and his colleagues, using splanchnic arterio–venous differences in humans, appeared to demonstrate that only 15 per cent of the ingested glucose was available for systemic metabolism. The remainder was ac-

counted for by a very large first-pass uptake by the liver as well as in substituting for a suppressed endogenous production of glucose (Felig et al., 1975). In type-2 diabetics, it also appeared that this first-pass uptake was attenuated, increasing the systemic delivery of glucose and thus diminishing glucose tolerance (Felig et al., 1978, Felig, 1980).

That this was not the case, however, was demonstrated using tracer methods in this laboratory (Radziuk et al., 1978 a). An intravenously infused tracer was used to monitor the systemic metabolic clearance of glucose. An oral glucose load labelled with a second tracer was then administered. The systemic appearance of the second tracer was monitored using the concentrations of both tracers in a mathematical model describing the kinetics of glucose. It was demonstrated that nearly 95 per cent of the glucose ingested in fact appeared in the peripheral circulation. This was an unequivocal demonstration of the relatively small degree of extraction of glucose by the liver, even under circumstances when this is given orally. Part of the explanation for this is that portal glucose is only approximately 20 per cent higher than arterial during absorption of such glucose load. This is because absorption takes place over at least 4 hours, contrary to what has been previously believed (1-2 hours). A 5-8 per cent first-pass uptake of ingested glucose by the liver, however, does not imply the uptake of a total amount of glucose equivalent to about 20-25 per cent of the ingested glucose load. This is not insignificant but also does not appear to be the major pathway for glucose disposal.

It was subsequently demonstrated (Radziuk & Inculet, 1983) that the improvement in oral glucose tolerance could be entirely attributed to increased uptake of glucose by muscle which in this series of studies was represented by the forearm. There was about a 50 per cent increase in the peripheral glucose uptake after oral glucose administration (relatively

to intravenous) reported in this study. This was moreover consistent with a large increase in insulin concentrations following oral glucose relative to intravenous when the loads were equivalent.

The increase in insulin following glucose load has long been known (Elrick et al., 1964; McIntyre et al., 1965). It is now generally attributed to the action of the entero-insular axis, as already discussed (Unger & Eisentraut, 1969). This axis consists of all the effects which could be attributed to the gut and which act on B-cells of the pancreas to stimulate secretion of insulin. Although neural influences may also be implicated (as mentioned above), in the present work we have focused on peptides secreted by endocrine cells of the gut. A brief overview of efforts in examining particular gut endocrine agents was presented in the Introduction of this thesis. As can be seen, a large number of gut peptides have been shown to stimulate the secretion of insulin. Most of these effects, however, have been pharmacological. Until recently, only gastric inhibitory polypeptide (GIP) has been demonstrated to be released in a dose-responsive fashion during the absorption of glucose (Dupre et al., 1973; Brown et al., 1975; Elahi et al., 1979). Also at physiological levels, GIP has been demonstrated to stimulate insulin secretion (Dupre et al., 1973; Brown et al., 1975). It appears, therefore, to be the first well-defined incretin. Work from the laboratories of Creutzfeldt (Ebert & Creutzfeldt, 1982; Creutzfeldt et al., 1983), Bloom (Bloom, 1974) and others (Sarson et al., 1982, 1984; Salera et al., 1983; Nauck et al., 1989), however, suggested that GIP alone is not sufficient to explain the incretin effect following meals or oral glucose loads. The relatively recent discovery of glucagon-like peptide-1 (GLP-1) is now almost certain to have added a second hormone of the incretin family.

GLP-1 is secreted from L-cells of the gut, which are primarily localized in the ileum (Ors-

kov et al., 1989; Namba et al., 1990; Thorens, 1992). This peptide has been shown in humans to be released in response to meals and glucose loads (Kreymann et al., 1987). Moreover, physiological concentrations have been shown to stimulate the release of insulin (Kreymann et al., 1987; Orskov et al., 1988). Interestingly, it has been suggested that GLP-1 has a number of other actions. These include the enhancement of insulin sensitivity (Montrose-Rafizadeh et al., 1994), suppression of glucagon (Kreymann et al., 1987; Orskov et al., 1988; Kawai et al., 1989; Komatsu et al., 1989) and slowing of gastric emptying during meals (Schjoldager et al., 1989; O'Halloran et al., 1990). Moreover, its actions have been suggested to include direct effects on both liver (Mommensen et al., 1987; Egan et al., 1994) and muscle (Richter et al., 1993) as well as the already mentioned improvement of insulin action.

It is evident that some or other of these actions may be more important under different physiological and pathophysiological situations. For example, the action of GLP-1 in diabetes may be quite different from that in normal physiology (Orskov et al., 1991; Gutniak et al., 1992; Nauck et al., 1993;). The purpose of this work, therefore, was to attempt to dissect out the actions of GLP-1 under normal physiological conditions and to examine the relative importance of the several actions which have been hypothesized. In order to do this, a pig model was chosen. Part of the rationale for this is that the pig appears to metabolize glucose loads in a manner similar to that of humans. The strategy was to analyze the systemic effects of GLP-1 by measuring arterial insulin and C-peptide concentrations to assess changes in insulin secretion while monitoring the amount of glucose which was necessary to maintain a hyperglycemic clamp during GLP-1 infusion (at two doses). This would indicate the rate of glucose disposal upon GLP-1 stimulation. Secondly, in order to distinguish between different tissue effects of GLP-1, these effects were separately measured in

the liver, the intestine and the hindlimb. This was accomplished by determinations of arterio-venous differences in hormone or metabolite concentrations, combined with the measurement of blood (or plasma) flow through the particular tissue bed. The doses used (4 ng/kg/min and 8 ng/kg/min) were chosen since similar infusion rates had been used in man to simulate the physiological excursions of GLP-1 seen after meals.

The results could be summarized as follows:

- (1) GLP-1 infusion stimulated insulin secretion approximately two-fold. This was indicated both by increases in insulin and C-peptide concentrations systemically as well as by increases in net splanchnic output of C-peptide. The splanchnic output of C-peptide is indicative of insulin secretion since, unlike insulin (Blackard & Nelson, 1970; Field, 1973; Eaton et al. 1980; Polonsky et al., 1983), C-peptide is not removed by the liver to a significant extent (Blackard & Nelson, 1970; Stoll et al., 1970; Eaton et al., 1980; Polonsky et al., 1983). The release of C-peptide from the splanchnic bed corresponds to its release from the pancreas, and therefore to the secretion of insulin which is equimolar with that of C-peptide.
- (2) The amount of glucose which is necessary to be infused to maintain the hyperglycemic clamp (150 mg/dl) doubled during the infusion of GLP-1 relative to control. This is indicative of increased glucose disposal while GLP-1 infusion progresses.
- (3) The transition of the liver from glucose production to glucose uptake was similar during GLP-1 infusion and the control studies.

- (4) The uptake of glucose by the intestinal bed was not different whether GLP-1 was infused or not.
- (5) Glucose uptake by the muscle during GLP-1 infusion was at least double that in the control situation, indicating that this is a primary site at which the systemic increase in glucose uptake is generated.
- (6) There was a marginally significant suppression of glucagon under the circumstances of the study. This was not different whether GLP-1 was infused or not.

These data enable us to focus on the principal physiological actions of GLP-1 among the many actions which have been hypothesized. Firstly, there does not appear to be any major hepatic effect, whether direct or mediated by the GLP-1 action on insulin or glucagon. This is consistent with the relatively minor action of the liver in the removal of postprandial glucose as discussed above. Since glucose or meal ingestion appears to make only a small or negligible difference in the hepatic uptake of glucose (Radziuk et al., 1978 a), there was little opportunity for a gut factor to increase this uptake under physiological circumstances. Similarly, although the intestine appears to be sensitive to increases in insulin and glucose, and increases its glucose uptake under the circumstances of these studies, again, there does not appear to be any direct or indirect effect of GLP-1 on this increased uptake. It is not expected that a major effect would be seen here, since the small first-pass effect which was measured using tracer studies (discussed above), would also include some degree of uptake by the entire splanchnic bed. It must be emphasized once again that this uptake is relatively small and leaves little room for normal effects in its enhancement.

This situation changes significantly in peripheral tissues. The similar pattern in hindlimb (muscle) of glucose uptake and the increase in the glucose infusion rate necessary to maintain hyperglycemia suggests, that muscle glucose uptake is in fact responsible for the systemic changes observed. These observations therefore focus on the muscle as the primary site of GLP-1 action.

Whether this action is direct or indirect is the remaining question. A direct action of GLP-1 would imply an action which is independent of insulin. A second direct action of GLP-1 would involve the enhancement of insulin action at the target tissue level. The indirect effects of GLP-1 would be mediated simply by its increasing insulin secretion over and above that which is resulting from the hyperglycemia alone. Insight into this question can be gained by comparing the increments in insulin concentration and those in the glucose infusion rate along with the glucose uptake by the muscle compared to control. It is seen in the results presented, that all these effects are approximately doubled. Since insulin concentrations are not elevated to the levels at which the insulin effect begins to saturate, it would be expected that a doubling of insulin concentrations (relative to control) would approximately double its effect. Since this is what was seen in the present study, the data strongly suggest that the principal action of GLP-1 is on insulin secretion, which then mediates further effects on glucose uptake. The lack of difference in lactate levels and in the peripheral (hindlimb) lactate production when GLP-1 is infused relative to control, suggests further that the major effect of the additional insulin is on glucose storage (i.e. glycogen). This again is consistent with the heavy recent emphasis on muscle glycogen as the site of improvement of the glucose tolerance and glycogen synthase as a potential site of problems in type-2 diabetes (Beck-Nielsen et al., 1992; Consoli, 1992).

Thus, the physiological action of GLP-1 (at least in the pig model) appears to be primarily in the enhancement of insulin secretion. The systemic effects of this peptide therefore appear to be primarily those of an incretin. Although other effects have been suggested (Valverde et al., 1993; D'Alessio et al., 1994), they are likely not primary and may be accentuated particularly under pathophysiological conditions such as type-2 diabetes. The data presented here are therefore consistent with this action on GLP-1 as an incretin as well as being consistent with the previously defined relative roles of the splanchnic and peripheral tissue beds in physiological glucose disposal following meals.

5. CONCLUSIONS

The results presented in this thesis allow the formulation of the following statements:

- (1) GLP-1 fulfils the criteria for incretin, since in the context of increase blood glucose concentrations, an increase in GLP-1 concentrations generated by intravenous infusion, induced a significant insulinotropic effect .**
- (2) This additional insulin appears to be the primary factor mediating the glucoregulatory effect of GLP-1.**
- (3) Further confirmation that GLP-1 action is mediated by insulin is found in the fact that its effects are mainly expressed at the level of peripheral tissue, where insulin usually exerts its major effects.**
- (4) Glucose uptake increased proportionally to rises in insulin concentrations. This indicates that there is little likelihood of any additional direct effects of GLP-1 or of modulation of insulin sensitivity by this peptide.**

6. REFERENCES

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